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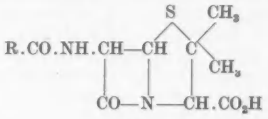
The Biosynthesis of Penicillin

1. THE INCORPORATION OF SOME AMINO ACIDS INTO PENICILLIN

By H. R. V. ARNSTEIN and P. T. GRANT
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 28 *October* 1953)

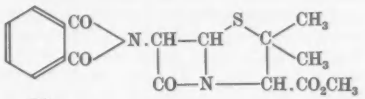
Although many useful antibiotics have been discovered in the last decade, penicillin appears still to be unique in combining extremely high antibacterial potency with negligible toxicity. The penicillin produced commercially is almost entirely benzylpenicillin (I, R = $C_6H_5.CH_2$—),



(I)

which is effective mainly against Gram-positive bacteria. Since structurally modified penicillins might show enhanced activity against other micro-organisms, many penicillin analogues have been prepared. These compounds, which differ only in the acyl group, RCO— (I), were obtained by biosynthetic methods (Behrens, 1949) or by chemical modifications of natural penicillins (Coghill, Stodola & Wachtel, 1949). Detailed information concerning the antibacterial activity and the therapeutic effectiveness of these penicillin derivatives is apparently not available (see, for example, Coghill *et al.* 1949), but it seems that none of them possesses properties greatly superior to those of benzylpenicillin. Indeed, the antibacterial activity of these modified penicillins against a benzylpenicillin-resistant strain of *Staphylococcus aureus* was not significantly greater than that of benzylpenicillin itself (Behrens & Kingkade, 1948). This evidence suggests that variation of the side-chain (R) does not markedly affect the antibacterial activity of penicillin.

The only other known analogue which still retains the fused β-lactam-thiazolidine rings of penicillin is a methyl penicillinate (II) synthesized by Sheehan, Henery-Logan & Johnson (1953). The fact that this compound is inactive against penicillin-sensitive bacteria suggests that the penicillin amide group is essential for biological potency. On this basis, the only remaining possibility of obtaining active penicillin analogues would be by substitution in the β-lactam-thiazolidine ring system. The preparation of such penicillins by biosynthesis is rendered difficult by an almost complete lack of knowledge of the metabolic pathways involved in penicillin biosynthesis.



(II)

Earlier studies of penicillin precursors (e.g. Behrens, 1949; Süs, 1950) were based on the assumption that the addition of a precursor to a penicillin-producing mould culture would increase the yield of penicillin. It is evident, however, that a precursor might not be effective in stimulating penicillin formation if it participated only in steps which are not rate-limiting under the particular experimental conditions. The problem of penicillin biosynthesis has therefore been re-investigated by the isotopic tracer technique. This method is particularly suited to such work, since considerations of rate-limiting steps are not essential to the interpretation of results. The present paper deals with the probable role of some amino acids in the biosynthesis of penicillin, and the following paper (Arnstein & Grant, 1954) describes experiments indicating that cysteine is a precursor of penicillin. Most of this work has been briefly reported in a preliminary communication (Arnstein & Grant, 1953).

EXPERIMENTAL

Fermentation methods

Preparation of spore inoculum. The organism used in all experiments was *Penicillium chrysogenum* WIS 48–701 (Johnson, 1952), which was obtained from Dr M. Lumb of Boots Pure Drug Co. Ltd., Nottingham. Spores were suspended in heat-treated horse serum and freeze-dried in ampoules which were then sealed and stored at 4°. From these master cultures, a spore inoculum was prepared essentially as described by Perret (1953). Before use, the inoculum was diluted to contain approx. 6×10^5 spores/ml., as determined by a direct count with a haemocytometer cell.

Fermentation apparatus. The fermentations (25 ml. medium) were carried out in 100 ml. micro-Kjeldahl flasks (Fig. 1). Each flask was fitted with a rubber-stoppered side arm used for inoculating the medium, making various additions and withdrawing samples for assay. The fermentation was aerated through a sintered glass disk (no. 3 porosity) by air which had been sterilized by passage through a cotton-wool filter. The effluent air from each flask was passed through another, smaller filter. CO_2 in the effluent air was retained by two traps containing 40% (w/v) NaOH (15 ml./trap). Foaming was effectively controlled by coating the neck of the flask with a thin film of silicone anti-foam (silicone DC anti-foam 'A', Midland Silicones Ltd., London), which was applied as a 5% (w/v) suspension in ether.

Each complete apparatus was fixed by Terry clips to an aluminium frame which was constructed to hold six flasks and the air filters. After being sterilized, the frame was mounted on a reciprocating shaker (3 in. throw at 240 strokes/min.).

Preparation of medium and details of fermentation. The basal synthetic fermentation medium, which had the composition given by Jarvis & Johnson (1950), was supplemented with 0·05% (w/v) Bacto yeast extract (Difco Laboratories Inc., Detroit, U.S.A.). Aqueous potassium phenylacetate (10%, w/v) was added every 24 hr.; the first addition was 35 hr. after inoculation. Each addition was calculated to give a final concentration of 0·12% (w/v) phenylacetic acid in the medium.

The inorganic constituents of the fermentation medium were steam-sterilized for about 20 min. at 15 lb./sq.in. All organic constituents were dissolved in water (with the exception of cystine, for which N-HCl was used), filtered through a Seitz filter and added aseptically to the fermentation flask.

Fermentations were carried out at 24±1°. The medium was inoculated with 1 ml. of the spore inoculum described above and aerated at a rate of 1 vol. air/vol. medium/min. The maximum yield of penicillin, as estimated by bioassay, was obtained in about 140 hr.

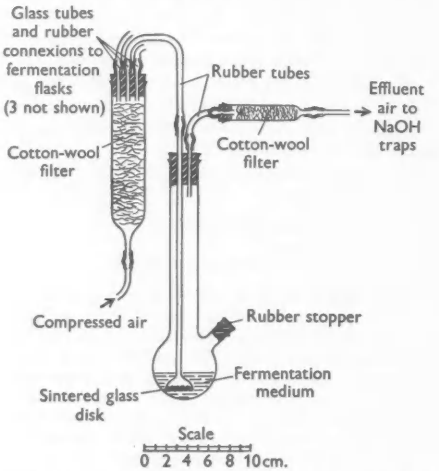


Fig. 1. Flask for small-scale fermentation. For further details see text.

Assay of penicillin. All bioassays were done by the cup-plate method (Humphrey & Lightbown, 1952) using *Bacillus subtilis* (I.C.I. strain, NCTC 8241) as test organisms and a solution of pure sodium benzylpenicillin as standard.

Isolation of penicillin. Since the total amount of penicillin in the above micro-fermentation was only about 1–4 mg. per flask, it was necessary to add non-radioactive carrier before isolating the penicillin. The fermented culture fluid was filtered to remove the mycelium, which was washed with 0·05 M potassium phosphate buffer (pH 6·9). The filtrate and washings were diluted to 50 ml. with the buffer and assayed. A solution of sodium benzylpenicillin (40 mg. in 4 ml.), which had been assayed at the same time as the diluted broth filtrate, was then added. By this procedure no weighing error is introduced and the dilution of the radioactive penicillin in the broth can be calculated independently of impurities in the carrier penicillin. The method depends, however, on the absence of penicillin other than benzylpenicillin. Using the method of Glister & Grainger (1950), the carrier benzylpenicillin was found to be chromatographically pure, while the experimentally produced penicillin contained only traces (<2%) of other penicillins.

The diluted penicillin was extracted from the solution with pentyl acetate after acidification to pH 2–2·5 with 50% (w/v) phosphoric acid and concentrated by successive distributions between ether and buffer (Lester Smith & Hockenhull, 1952). Finally, the free acid in dry ether (10 ml.) was precipitated as the *N*-ethylpiperidine salt, which was recrystallized to constant radioactivity from chloroform–acetone.

Radioactivity measurements

All measurements of radioactivity were carried out with a helium-filled bell-shaped Geiger-Müller counter, using 'infinite thickness' samples mounted on 1 sq.cm. Polythene disks (Popják, 1950). All counts were compared with a ^{14}C-Perspex standard, obtained from the Radiochemical Centre, Amersham. The standard error in all determinations was <5%.

Labelled compounds

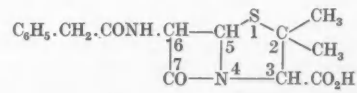
[α-^{14}C]Glycine and DL-[γ-^{14}C]valine were obtained from the Radiochemical Centre, Amersham. DL-[β-^{14}C]Cystine was prepared by Dr J. C. Crawhall (Arnstein & Crawhall, 1953; cf. Crawhall & Elliott, 1951). The labelled cystine was resolved by the isotopic dilution technique of Wood & Gutmann (1949). DL-[β-^{14}C]Serine was synthesized as described by Arnstein (1951).

Isolation of glycine and serine from mycelium

In one experiment (Table 1, no. 1) the mycelium was hydrolysed with 6 N-HCl at 105° for 24 hr. in a sealed tube. Glycine and serine were isolated as the dinitrophenyl derivatives, essentially as described by Campbell & Work (1952).

Degradation of penicillin

Numbering of atoms. The following numbering of penicillin, which is that given by Sheehan *et al.* (1953), has been adopted:



Method. Penicillin was degraded chemically in order to locate the distribution of radioactivity in the penicillamine and penilloaldehyde moieties of the molecule. In most experiments, the penilloaldehyde was degraded further and the radioactivity in carbon atoms 5–7 was determined separately. Most of the chemical reactions involved in this method have been outlined by Clarke, Johnson & Robinson (1949). The following is a typical degradation.

Benzylpenicillin *N*-ethylpiperidine salt (394 mg., 0·88 m-mole) in water (5 ml.) was cooled to 0° and the free acid extracted into ether (2 × 10 ml.) at pH 2·5. The ether solution was shaken with ice-cold 0·1 M sodium phosphate buffer (2 × 5 ml., pH 6·9) and the aqueous solution of sodium benzylpenicillin separated, made 0·1 N with respect to H_2SO_4 and heated for 3 hr. at 100° in a current of CO_2-free N_2. The evolved CO_2 was absorbed in sat. aqueous $Ba(OH)_2$; the precipitated $BaCO_3$ was washed with water, ethanol and ether and dried (124 mg., 78%). The radioactivity of the $BaCO_3$ represents the amount of ^{14}C at $C_{(7)}$ of penicillin.

The above solution of penilloic acid was adjusted to pH 6·5 with 2 N-NaOH and 10 ml. sat. aqueous $HgCl_2$ were slowly added with stirring. The dense white ppt. of penicillamine mercaptide was coagulated by stirring at 40° for a few minutes, centrifuged and washed repeatedly with sat. aqueous $HgCl_2$ till the supernatant ceased to give a ppt. with 2:4-dinitrophenylhydrazine. After being washed with ethanol and ether, it was dried (yield of crude material 195 mg., 90%). The mercaptide was suspended in 0·1 N-HCl (15 ml.) and treated with H_2S. HgS was filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was boiled for 5 min. with dry acetone (10 ml.) containing 1 drop of conc. HCl. The hot solution was filtered and the flask was washed with more acetone (5 ml.). The filtrate was concentrated to 2 ml. when isopropylidenepenicillamine hydrochloride (79 mg., 40%) crystallized. It was recrystallized from hot acetone or from acetone–ligroin, giving material of constant radioactivity, m.p. 192°.

The supernatant solution remaining after precipitation of the penicillamine mercaptide was immediately cooled in ice and treated with H_2S. HgS was filtered off and H_2S was removed from the filtrate with a stream of air. A portion of the filtrate (20% of the total volume) was added to a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl, yielding crude benzylpenilloaldehyde 2:4-dinitrophenylhydrazone (31 mg., 49%; m.p. 171°), which was recrystallized from ethanol to give the pure product, m.p. 192–193°. The bulk of the above solution containing penilloaldehyde was cooled to 0° and a fivefold excess of freshly prepared ammoniacal Ag_2O solution (from 1·16 g. Ag_2O and 6 ml. conc. ammonia, sp.gr. 0·88) was added. The mixture was shaken for 12 hr. at 4°, acidified with conc. HCl and filtered. The filtrate was evaporated to dryness *in vacuo*, the residue was dissolved in 6 N-HCl (10 ml.) and the solution was heated for 12 hr. at 110° in a sealed tube. After evaporation to dryness *in vacuo* the residue was extracted with ether to remove phenylacetic acid. The remaining material was dissolved in ethanol, the solution was filtered and pyridine was added to the filtrate giving glycine (18 mg., 27%), which was shown to be free from other amino acids by chromatography on paper. The glycine was further degraded with ninhydrin to formaldehyde, which was isolated as the dimedone derivative (m.p. 189°), and CO_2, which was converted into $BaCO_3$ (Arnstein, 1951). The radioactivities of the $BaCO_3$ and formaldehyde dimedone corresponds to the amount of ^{14}C in $C_{(5)}$ and $C_{(6)}$ of penicillin, respectively.

RESULTS

The utilization of glycine, valine, serine and cystine for penicillin biosynthesis. The penicillin isolated from fermentations to which ^{14}C-labelled glycine, valine, serine or cystine had been added contained significant amounts of ^{14}C in all cases (Table 1). Of

Table 1. *Conversion of* [α-^{14}C]*glycine*, DL-[γ-^{14}C]*valine*, DL-[β-^{14}C]*serine and* DL-[β-^{14}C]*cystine into penicillin*

With the exception of Expt. no. 3, fermentations were carried out in duplicate. In Expts. nos. 1, 2 and 4, the amino acids were added at zero time, 18, 41, 65, 90 and 114 hr. as equimolar solutions (0·5 ml., approx. 0·4 μmole/ml. broth/addition). Expt. no. 3 was a comparable large-scale fermentation (1·5 l.) as described by Arnstein & Grant (1954). Cystine was added in N-HCl, followed by an equivalent amount of N-KOH, the other amino acids were added as aqueous solutions; all solutions were sterilized by Seitz filtration. The pure sodium benzylpenicillin used as standard assayed at 1650 units/mg. For other experimental details see text.

Expt. no.	Amino acid added	mg.	μc	Yield of penicillin (units)	Radioactivity of penicillin μc*	Radioactivity of penicillin % of added ^{14}C	Specific radioactivity (μc/m-mole) Amino acid added (*A*)	Specific radioactivity (μc/m-mole) Penicillin (*B*)	Dilution of molar radioactivity (*A*/*B*)
1	[α-^{14}C]Glycine	4·92	1·82	4 800	0·019	1·0	27·8	2·30	12·1
				6 650	0·019	1·0	27·8	1·68	16·6
2	DL-[γ-^{14}C]Valine	7·02	1·56	3 700	0·026	1·7	26·1	4·16	6·3
				2 750	0·023	1·5	26·1	4·85	5·4
3	DL-[β-^{14}C]Serine	378·5	1·96	300 000	0·054	2·8	0·543	0·105	5·2
4	DL-[β-^{14}C]Cystine	7·47†	3·08	2 200	0·057	1·8	56·5‡	15·2	3·7
				3 600	0·106	3·4	56·5‡	17·2	3·3

* Calculated from the specific radioactivity and the total amount of penicillin produced.

† 4·89 mg. (3·08 μc) DL-[β-^{14}C]cystine + 2·58 mg. non-radioactive L-cystine.

‡ Calculated as μc/m-mole cysteine. The figure is an average of the specific radioactivity of the L- and D-cystine, which was 37·2 and 76·2 μc/m-mole cysteine, respectively.

the four amino acids investigated, cystine appears to be the best penicillin precursor, as shown by the relatively high incorporation of ^{14}C into penicillin and by the low dilution of the molar radioactivity. Serine and valine were utilized with approximately similar dilutions, which are, however, somewhat higher than those in the cystine experiments. Glycine is the least efficient precursor, the dilution of molar radioactivity in this case being about 4 times that in the cystine experiments. Table 1 also shows the penicillin yields obtained in these fermentations. In control experiments with micro-fermentations, titres of about 150 units/ml. were usually obtained and it seems, therefore, that the addition of glycine or DL-valine had little effect on the penicillin yield. DL-Cystine may have caused a slight decrease in penicillin formation, but in view of the small number of experiments this observation may not be statistically significant. The serine experiment was a large-scale fermentation (Arnstein & Grant, 1954) in which somewhat higher penicillin titres (200–400 units/ml.) were usually obtained.

Location of radioactivity in biosynthetic penicillin. The distribution of ^{14}C in the penicillin derived from [α-^{14}C]glycine, DL-[γ-^{14}C]valine and DL-[β-^{14}C]-cystine (Table 2) indicates clearly that valine is incorporated only into the penicillamine moiety of penicillin and that glycine and cystine are converted into the β-lactam portion of the molecule. The α-labelled glycine gave rise to penicillin containing ^{14}C chiefly at $C_{(6)}$, although about 3% of the total radioactivity was located at $C_{(5)}$. In this experiment, glycine and serine of the mycelial protein were isolated as the 2:4-dinitrophenyl derivatives. It was found that the specific radioactivity of the serine was about one-half that of the glycine.

[β-^{14}C]Cystine gave rise to penicillin containing 95% of the total radioactivity at $C_{(5)}$, which is the carbon atom common to the thiazolidine and β-lactam rings.

In all experiments, about 95% of the radioactivity present in the penicillin has been accounted for in the degradation products; the remaining 5% was not assignable to any individual carbon atom, but in view of the difficulty of measuring very low radioactivity it is possible that some or all of the positions designated as 'inactive' in Table 2 did in

Table 2. *Distribution of radioactivity in penicillin obtained from fermentations with labelled amino acids*

Details of the fermentations are given in Table 1. The penicillin from duplicate fermentations was combined, diluted with carrier and degraded as described in the Experimental section. The percentage radioactivity in separate carbon atoms of penicillin = 100 a/b where a is the molar radioactivity of the appropriate degradation product and b the molar radioactivity of penicillin after dilution with carrier.

Degradation product	Atom number in thiazolidine-β-lactam rings of penicillin*	Distribution of radioactivity in penicillin carbon atoms (%)		
		Glycine expt.	Valine expt.	Cystine expt.
$BaCO_3$ from acid-labile CO_2	7	Inactive	Inactive	Inactive
Isopropylidenepenicillamine HCl	(2 + 3)†	Inactive	95·2	Inactive
Benzylpenilloaldehyde 2:4-dinitrophenylhydrazone	5 + 6	—	Inactive	94·5
$BaCO_3$ from glycine carboxyl carbon	5	2·6	—	96·1
Formaldehyde dimedone from glycine α-carbon	6	94·1	—	Inactive

* See Experimental section for numbering of penicillin.
† Includes also the *gem*-dimethyl groups at $C_{(2)}$ and the carboxyl group at $C_{(3)}$.
— Signifies not determined.

Table 3. *Conversion of the optical enantiomorphs of* [β-^{14}C]*cystine into penicillin, respiratory carbon dioxide and mycelium*

In duplicate experiments, D- or L-[β-^{14}C]cystine was added daily in equal amounts (40 μg./ml. broth, i.e. 1 mg./flask/day) starting at zero time. The amino acids were added as Seitz-filtered solutions in N-HCl, each addition being followed by an equal amount of N-KOH. CO_2 in the effluent air was absorbed in 40% NaOH as described in the Experimental section and counted as $BaCO_3$. At the end of the fermentation, the mycelium was filtered off, washed with water and dried. After extraction with ether, it was powdered and assayed directly for ^{14}C.

Amino acid added	Radioactivity (μc)			Specific radioactivity (μc/m-mole)		Dilution of molar radioactivity (A/B)
	Respiratory CO_2	Fat-free mycelium	Penicillin	Cystine added* (A)	Penicillin (B)	
L-Cystine	0·08	0·068	0·0029	3·05	1·38	2·21
	0·07	0·071	0·0036	3·05	0·88	3·46
D-Cystine	0·024	0·022	0·00046	3·28	0·22	14·9
	0·022	0·025	0·00145	3·28	0·25	13·1

* Calculated as μc/m-mole cysteine.

fact contain traces of ^{14}C. It is unlikely, however, that more than 1–2 % of the total radioactivity was present in any one of these carbon atoms.

The stereochemical specificity of the conversion of cystine into penicillin. In view of the relatively efficient incorporation of DL-cystine into penicillin, it was of interest to investigate the stereochemical specificity of this reaction. Carbon-atom 6 of penicillin has been shown to have the same configuration as L-alanine (Kaczka & Folkers, 1949). It was expected that L-cystine would therefore be a better penicillin precursor than the D-enantiomorph. The results given in Table 3 show clearly that this was indeed the case, L-cystine being about 5 times more efficient as a precursor than D-cystine. It is of interest that the general metabolism of the two enantiomorphs, such as their conversion into respiratory carbon dioxide or into the mycelia protein, appears to correspond fairly closely to their utilization for penicillin biosynthesis. These results are compatible with the possibility that D-cystine is not metabolized as such by the mould but is converted into the L-enantiomorph, although only to a limited extent.

Incorporation of DL-cystine into penicillin when added at various times during the fermentation. In previous experiments, the labelled compounds were added at intervals throughout the fermentation in order to obtain penicillin with a relatively high specific radioactivity. Although this was achieved, only about 1–3 % of the total amount of precursor was converted into penicillin (Table 1). It was thought that a more favourable utilization of the precursor might take place if a single addition of a smaller quantity were made at the most suitable stage of the fermentation, i.e. at the peak of penicillin biosynthesis. DL-[β-^{14}C]Cystine was therefore added at various times to a series of similar fermentations (Table 4). It can be seen that the best incorporation was obtained when the precursor was added at 23 or 47 hr. It may be significant that this time appears to coincide with the beginning of active amino acid metabolism, as shown by the extensive conversion of glycine, valine or cystine into respiratory carbon dioxide (Fig. 2). The decline in the incorporation of cystine into penicillin during later stages of the fermentation was less marked than expected, and a significant utilization of

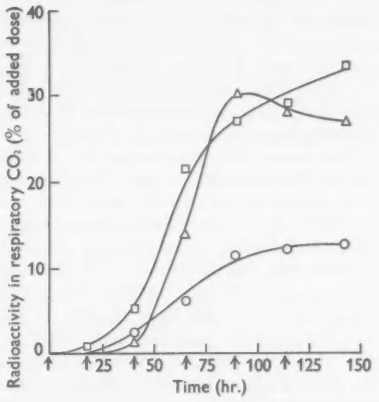


Fig. 2. Conversion of glycine, valine and cystine into respiratory carbon dioxide in penicillin fermentations. [α-^{14}C]Glycine, DL-[γ-^{14}C]valine and DL-[β-^{14}C]cystine were added to fermentations as described in Table 1, each addition being 0·303, 0·260 and 0·513 μc, respectively. Respiratory carbon dioxide was collected over various time intervals (0–18, 18–41, 41–65, 65–90, 90–114 and 114–142 hr.) during the fermentation and converted into $BaCO_3$, which was weighed and assayed for ^{14}C. The times at which the labelled amino acids were added are indicated by arrows. The amount of added amino acid converted into carbon dioxide (x) at various periods of the fermentation was derived from the formula, $x = 100\,a/b$, where a is the amount of ^{14}C in all the $BaCO_3$ fractions collected from the beginning of the fermentation till the stated time, and b is the amount of ^{14}C in the additions of labelled amino acid made before the stated time. □—□, Glycine expt.; △—△, valine expt.; ○—○, cystine expt.

Table 4. *Utilization of* DL-[β-^{14}C]*cystine for penicillin biosynthesis when added at various times during the fermentation*

DL-[β-^{14}C]Cystine (0·716 mg., 0·452 μc) in 0·1 ml. N-HCl was added to fermentations at the times shown below. At the end of each fermentation (121 hr.) the penicillin was isolated after dilution with carrier as described in the Experimental section.

Time of addition of DL-[β-^{14}C]-cystine (hr.)	Radioactivity in penicillin		Specific radioactivity (μc/m-mole)		Dilution of molar radioactivity (A/B)
	μc	% of added ^{14}C	Cystine (A)	Penicillin (B)	
0	0·012	2·7	76·2*	1·72	44·3
23	0·018	4·0		2·88	26·4
47	0·019	4·2		2·66	28·6
71	0·014	3·1		2·07	36·8
95	0·008	1·8		1·29	59·0

* Calculated as μc/m-mole cysteine.

precursor was observed even when the amino acid was added at 95 hr., i.e. 26 hr. before the end of the fermentation (Table 4).

DISCUSSION

Early speculations concerning the biosynthesis of penicillin postulated the participation of three intermediates, phenylacetic acid, aminomalonsemialdehyde and penicillamine (Behrens, 1949). Phenylacetic acid may be formed from phenylalanine, since both compounds stimulate penicillin biosynthesis. Aminomalonsemialdehyde could presumably originate from serine, although this reaction has never been demonstrated. It is noteworthy that the presence of penicillamine in penicillin fermentations has also not been shown and no work on the biosynthesis of this amino acid has been reported. An alternative mechanism of penicillin biosynthesis was proposed later by Hockenhull, Ramachandran & Walker (1949) who suggested a condensation of D-β-hydroxyvaline, L-cysteine and a carboxylic acid, such as phenylacetic acid in the case of benzylpenicillin. However, no experimental work supporting this hypothesis was reported by these authors and until recently there has been no evidence concerning the origin of the thiazolidine-β-lactam rings of penicillin or the participation of amino acids in the biosynthesis. On the contrary, an experiment with deuteriophenylacetyl-DL-[^{15}N]valine appeared to indicate that valine was not used for penicillin biosynthesis (Behrens, 1949; see also Behrens *et al.* 1948). In other experiments, it was found that the phenylacetyl derivatives of DL-valine, DL-serine, DL-cysteine and glycine, as well as those of other amino acids, were not superior to phenylacetic acid or phenylacetamide in increasing penicillin production. Since hydrolysis of the phenylacetyl derivatives must have occurred, the above evidence indirectly suggests that glycine, serine, valine or cystine do not stimulate penicillin formation. Similar results were obtained in experiments using serine, valine or cystine (Behrens, 1949).

The present experiments demonstrate that glycine, serine, valine and cystine participate in the biosynthesis of the penicillin molecule, although they have little or no effect on the amount of penicillin formed. Thus the daily addition of the above amino acids, labelled with ^{14}C, to penicillin fermentations resulted in the incorporation of about 1–3·5% of the added radioactivity into the penicillin (Table 1). The apparently low incorporation of radioactivity into penicillin is probably due to the fact that the amount of penicillin formed is quantitatively insignificant when compared with the amount of mycelial protein. In agreement with this explanation, it was found that the radioactivity in the mycelium was about 20 to 50 times that in the penicillin (Table 3). Moreover, a large proportion of the added amino acids is converted into respiratory carbon dioxide (Fig. 2 and Table 3), and the amount of amino acid available for penicillin formation is therefore considerably less than that added to the medium.

The efficiency of incorporation of precursor also depends on the time of its addition to the fermentation. Thus the utilization of cystine was found to be optimum at 23–47 hr. after inoculation of the medium (Table 4). At these times a single dose was used somewhat more efficiently for penicillin biosynthesis than a larger amount added throughout the fermentation (Table 1). The total amount of ^{14}C incorporated into penicillin is therefore not a suitable criterion of precursor efficiency. On the other hand, the dilution of the molar radioactivity of the precursors indicates their relative importance, since, in a series of reactions A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ product, the specific radioactivity of the most immediate precursor, C, will be diluted least, provided of course that the experimental conditions, such as the amount of precursor added and the times of addition, are comparable. On this basis, cystine appears to be a better penicillin precursor than valine, serine or glycine (Table 1).

Degradation of the penicillin obtained in the valine, glycine and cystine experiments showed clearly that valine is a source of the penicillamine moiety of penicillin, whereas the other two amino acids contribute to the biosynthesis of the β-lactam portion (Table 2). It is likely that glycine and serine are incorporated into penicillin only after conversion into cystine. Glycine is known to be a serine precursor both in intact rats (Sakami, 1948) and in suspensions of rat liver (Winnick, Moring-Claesson & Greenberg, 1948). A similar reaction evidently takes place in the mould used in the present work, since radioactive serine was isolated from the mycelium of the fermentation to which [α-^{14}C]glycine had been added. The formation of cysteine from serine has been shown to occur in rat liver tissue (Binkley & du Vigneaud, 1942) and probably also in the intact rat (Stekol, Weiss & Weiss, 1950).

Evidence indicating that cystine, or a close derivative of this amino acid, may be used intact for penicillin biosynthesis is provided by the difference in utilization of the D- and L-enantiomorphs (Table 3). L-Cystine, which has the same configuration as the relevant carbon atom (position 6) of penicillin (Kaczka & Folkers, 1949) was converted into penicillin with an average dilution of molar radioactivity of only 2·8 compared with 14·0 for the corresponding value in the D-cystine experiment.

Recently, it has been reported that [^{14}C]formate is incorporated into penicillin, although the position of the radioactive atoms in the penicillin molecule

was not determined (Martin *et al.* 1953). It seems likely that formate condenses with glycine to give serine and thence cysteine, and that this pathway accounts for the conversion of formate into penicillin.

It has also been shown that the addition of L-cysteine or methionine (which can serve as a source of cystine sulphur as demonstrated both in the animal organism (Tarver & Schmidt, 1939) and in the mould *Aspergillus nidulans* (Hockenhull, 1949)) to fermentations containing radioactive sulphate reduced the amount of radioactivity found in the penicillin without affecting the penicillin yield (Stevens, Vohra, Inamine & Roholt, 1953). These experiments indicate, in general agreement with the present work, that cysteine is a more direct penicillin precursor than sulphate.

It is concluded that the fused thiazolidine-β-lactam portion of penicillin is probably synthesized by a condensation of cysteine, or a close derivative, with valine, or a related amino acid such as hydroxy- or dehydro-valine. More conclusive evidence showing that cysteine is incorporated intact into penicillin and that the observed dilution of the specific radioactivity of the added labelled cystine is due to endogenous cysteine synthesis by the mould is presented in the following paper (Arnstein & Grant, 1954).

SUMMARY

1. The conversion of [α-^{14}C]glycine, DL-[β-^{14}C]-serine, DL-, D- and L-[β-^{14}C]cystine and DL-[γ-^{14}C]-valine into penicillin has been studied by adding the labelled amino acids to fermentations of *Penicillium chrysogenum* WIS 48–701.

2. Valine was found to be a precursor of the penicillamine moiety of penicillin, whereas cystine and to a lesser extent glycine were incorporated into the β-lactam ring of the molecule.

3. Penicillin biosynthesis from cystine appears to be stereochemically specific, L-cystine being utilized to a far greater extent than the D enantiomorph.

4. The highest incorporation of radioactivity from cystine into penicillin was obtained when the amino acid was added to the fermentation at 23–47 hr. after inoculation.

5. The slight dilution of the specific radioactivity of cystine during its conversion into penicillin suggests that cysteine may be an important and direct precursor of penicillin.

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The Biosynthesis of Penicillin

2. THE INCORPORATION OF CYSTINE INTO PENICILLIN

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In the preceding paper (Arnstein & Grant, 1954) it was shown that [β-^{14}C]cystine is utilized by the mould *Penicillium chrysogenum* for penicillin formation. It was found that the radioactivity in the penicillin molecule was located almost entirely at position 5, which is the carbon atom common to the thiazolidine and β-lactam rings. Furthermore, L-cystine, which has the same configuration as the relevant carbon atom (6) of penicillin was a far better precursor than the D enantiomorph. It seemed likely, therefore, that cystine, after reduction to cysteine, is used directly for penicillin biosynthesis. However, the possibility of a less direct utilization of cystine, involving the formation of other intermediates, could not be excluded. In particular, since the above experiments were carried out with cystine labelled only with isotopic carbon, the results are also compatible with an initial conversion of cysteine into β, β-dimethyllanthionine, cleavage of this intermediate to penicillamine and serine and subsequent condensation of these two amino acids to give the thiazolidine-β-lactam ring structure of penicillin.

It was expected that further information about the pathway of penicillin biosynthesis might be obtained by using cystine labelled with isotopic nitrogen and sulphur as well as carbon. The present experiments with D- and L-[β-^{14}C, ^{35}S, ^{15}N]cystine, some of which have been briefly described in a preliminary communication (Arnstein & Grant, 1953), indicate that cysteine is used intact for penicillin formation.

EXPERIMENTAL

Fermentations

Organism and preparation of spore inoculum. Details of the strain of *Penicillium chrysogenum*, the preparation and storage of the master cultures have been described (Arnstein & Grant, 1954). Subcultures were prepared by inoculating molasses agar with spores from the master cultures (Perret, 1953). After 10 days at 24°, the spores growing on the mycelial felt (36 sq.in. area) were washed off with 40 ml. of 1% Teepol (Shell Chemicals Ltd., London) in 0·1 M potassium phosphate buffer (pH 6·9). After centrifuging, the spores were resuspended in fresh buffer (25 ml.) and used as inoculum of the fermentation medium.

Fermentation apparatus. The design was essentially that described by Brown & Peterson (1950), but the apparatus was scaled down to hold 1–1·5 l. of medium. In order to avoid leakage of effluent air, which contained $^{14}CO_2$, into the atmosphere, a seal running in liquid paraffin was attached to the propeller drive shaft. Aeration of the medium was carried out through a ring sparger. The incoming air was sterilized by passage through a cotton-wool filter (23 × 4·5 cm. diameter). The effluent air was passed through a second cotton-wool filter (10 × 4 cm. diameter). CO_2 was absorbed by 40% (w/v) NaOH (2 × 50 ml./day) contained in two gas-scrubbing bottles.

Details of medium and fermentations. The basal medium used was the synthetic medium of Jarvis & Johnson (1950), supplemented with 0·05% Bacto yeast extract (Difco Laboratories Inc., Detroit, U.S.A.). The inorganic salts were dissolved in water and steam-sterilized in the fermentation vessel, which had been connected to the air filters, at 20 lb./sq.in. for approx. 15 min. The organic constituents were Seitz-filtered and added aseptically followed by the inoculum. Ammonium phenylacetate (10·5% (w/v) in water) was added to a final concentration in the fermentation medium of 0·2% (w/v) every 24 hr. starting 36 hr. after inoculation. The fermentation was carried out at 24 ± 1°. The solution was stirred at 540 rev./min. and aerated with sterile air at the rate of 1 vol. air/vol. medium/min. Foaming was controlled by adding a steam-sterilized aqueous silicone emulsion (compound F4003, Midland Silicones Ltd., London) when required.

Bioassay. The cup-plate method with *Bacillus subtilis*, I.C.I. strain (NCTC 8241), as test organism (Humphrey & Lightbown, 1952) was used. When penicillin *N*-ethylpiperidine salt was assayed, a sample of pure benzylpenicillin which had been converted into the *N*-ethylpiperidine salt and recrystallized three times from chloroform–acetone was used as standard.

Isolation and degradation methods

Isolation and purification of penicillin. At the end of the fermentation, usually about 130–140 hr., the broth was cooled in ice and the pH was adjusted to 7 with 50% (w/v) phosphoric acid. The mycelium was filtered off and washed thoroughly with 0·05 M potassium phosphate buffer (pH 6·9). The filtered broth and washings were adjusted to pH 2–2·5 with 50% (w/v) phosphoric acid, penicillin was extracted with pentyl acetate and concentrated by successive extractions between ether and buffer (Lester Smith & Hockenhull, 1952), all operations being carried out at 4°. Finally, *N*-ethylpiperidine was added to a solution of the free acid in dry ether (10 ml.) and the *N*-ethylpiperidine salt was recrystallized from chloroform–acetone to constant radio-

activity. Usually, the yield was 50–60% of the penicillin present in the broth as calculated by bioassay. The purity of the product was estimated by its biological potency and by the inverse isotopic dilution method (cf. Radin, 1947), i.e. by measuring its radioactivity after accurate dilution of a small portion with pure nonradioactive benzylpenicillin *N*-ethylpiperidine salt.

Degradation of penicillin. The numbering of the thiazolidine-β-lactam rings of penicillin and the essential details of the degradation method have been given in the preceding paper (Arnstein & Grant, 1954). However, some modifications in the degradation procedure were necessary in order to locate and estimate the radioactivity due to ^{35}S and the relative abundance of the ^{15}N.

To determine whether the radioactivity of the penicillamine portion was wholly associated with the sulphur atom, the radioactivity of isopropylidenepenicillamine hydrochloride was measured directly and compared with that of the sulphur, which was converted into benzidine sulphate as described below.

The relative abundance of ^{15}N was measured on the nitrogen of the isopropylidene derivative of penicillamine and the 2-naphthalenesulphonyl derivative of glycine. The glycine was obtained by further degradation of penilloaldehyde and is derived from $C_{(5)}$, $C_{(6)}$ and the side-chain N of penicillin. The 2-naphthalenesulphonyl derivative (Abraham, Baker, Chain & Robinson, 1943) was recrystallized from hot water to constant radioactivity and also used to estimate the ^{14}C in $(C_{(5)} + C_{(6)})$ of penicillin.

Cystine and serine from mycelium. In some of the present experiments cystine was isolated as cysteic acid and the β-carbon atom of serine as the formaldehyde dimedon by the following procedures. Dried mycelium (17 g.) was continously extracted with ether for 24 hr. The mycelium was then extracted three times with 6% (w/v) trichloroacetic acid at 100°; the remaining solid was extracted with boiling ethanol and ether and dried at 80°. The dried residue (8 g.) was hydrolysed with a mixture of equal volumes of 12 N-HCl and formic acid (50 ml.) for 24 hr. at 105°. Excess of acid was removed *in vacuo* and the amino acids were precipitated with $HgCl_2$ (Campbell & Work, 1952; cf. Neuberg & Kerb, 1912) and regenerated from the ppt. with H_2S. The solution was made normal with respect to HCl, warmed to 60° and treated with an excess of sat. aqueous Br_2. After being stirred for 20 min. cystine had been oxidized completely, as shown by a negative nitroprusside test for sulphydryl groups. Excess of bromine was removed by evaporating the solution *in vacuo*. The residue was dissolved in water and the filtered solution was percolated through a column of 60 g. (wet wt.) Zeo-Karb 225 (Permutit Co. Ltd., London) in the hydrogen form. The column was developed with glass-distilled water, the effluent being collected in 7 ml. fractions. Cysteic acid was identified in fractions 4–7 by paper chromatography. No other amino acids were present in these fractions. The appropriate fractions were bulked and in some cases the cysteic acid was estimated quantitatively by the ninhydrin method of Moore & Stein (1948). The yield was approx. 3 mg./g. dry mycelial residue. After addition of non-isotopic carrier the cysteic acid was isolated as the monohydrate by addition of ethanol to a concentrated aqueous solution of the amino acid. On drying at 100° *in vacuo*, anhydrous cysteic acid was obtained. The radioactivity of this material was unchanged by subsequent recrystallization.

After the elution of cysteic acid from the Zeo-Karb column a further 150 ml. water was passed through in order to elute any remaining carbohydrate. The retained amino acids were then eluted with 0·2 M aqueous NH_3 (300 ml.) and the eluate concentrated to dryness *in vacuo*. This mixture, which contained no detectable reducing sugar (such as glucosamine, which might have remained on the column together with the amino acids), was oxidized with periodate (Rees, 1946). The formaldehyde arising from the β-carbon atom of serine was isolated as the dimedon derivative (cf. Arnstein & Neuberger, 1953).

Estimation of the ^{14}C, ^{35}S *and* ^{15}N *isotopes*

Radioactivity measurements. All measurements were carried out with a helium-filled, bell-shaped Geiger-Müller counter, using 'infinite thickness' samples (Popják, 1950). The substances were mounted on 1 sq.cm. Polythene disks or in the case of benzidine sulphate on filter paper (Whatman no. 50) on a 1 sq.cm. perforated stainless steel disk. All counts were compared with those of a ^{14}C-Perspex reference standard obtained from the Radiochemical Centre, Amersham. Radioactivity measurements of ^{35}S were corrected for decay by direct comparison with a subsidiary ^{35}S-standard.

For the assay of ^{35}S as benzidine sulphate enough material to give at least 25 mg. benzidine sulphate (3 mg. S) was digested with Pirie's reagent (Pirie, 1932). The subsequent procedure was essentially similar to that described by Simpson & Tarver (1950), except that the precipitation of benzidine sulphate was carried out in a much smaller volume (2–3 ml.).

Determination of ^{15}N. For ^{15}N assay, samples containing not less than 1 mg. N were digested by the micro-Kjeldahl procedure. The NH_3 was distilled, absorbed in 0·02 N-HCl (5 ml.) and converted into N_2 with alkaline hypobromite (Bentley, 1952; Sprinson & Rittenberg, 1949). Excess of ^{15}N was estimated in a 60° mass spectrometer.

Synthesis of labelled compounds

(All m.p.'s were determined on a micro block and are uncorrected.)

S-*Benzyl*-DL-[^{15}N]*cysteine.* Potassium phthalimide (5 g., 30 atom % ^{15}N excess), freshly distilled ethyl bromomalonate (7·1 g.) and dry xylene (50 ml.) were refluxed at 110–120° for 2 hr. and then heated at 140–145° for 1·5 hr. After 12 hr. at 4°, the solution was filtered and the residue was extracted with cold benzene (2 × 5 ml.). The washed residue was discarded and the filtrate and washings were combined. The solution was evaporated to dryness yielding a yellow oil which crystallized on standing. After addition of non-isotopic ethyl phthalimidomalonate (6·27 g.), the product was recrystallized from ether by adding light petroleum (b.p. 60–80°). The diluted ethyl [^{15}N]phthalimidomalonate (11·38 g.) was dissolved in dry ethanol, the solution was cooled quickly by immersion of the flask in liquid air and a solution of sodium (0·92 g.) in dry ethanol (20 ml.) was added simultaneously. The solvent was removed *in vacuo* giving the sodium derivative of ethyl [^{15}N]phthalimidomalonate, which was condensed with benzylthiomethyl chloride by the method of Wood & du Vigneaud (1939*a*). The product, ethyl benzylthiomethyl-[^{15}N]phthalimidomalonate, on hydrolysis and decarboxylation gave *S*-benzyl-DL-[^{15}N]cysteine (3·13 g., 32% based on the

potassium [^{15}N]phthalimide used; m.p. 217–218°, 14·2 atom % ^{15}N excess). From the mother liquors ^{15}N was recovered as $^{15}NH_3$ by Kjeldahl digestion and distillation.

*S-Benzyl-N-formyl-*DL*-[β-^{14}C or ^{15}N]cysteine.* DL-[β-^{14}C]-cystine, which had been synthesized by Dr J. C. Crawhall (Arnstein & Crawhall, 1953), and *S*-benzyl-DL-[^{15}N]cysteine were converted into the *S*-benzyl-*N*-formyl derivative as described by Wood & du Vigneaud (1939*b*).

N, S-*Diacetyl-*DL*-[^{35}S]cysteine.* [^{35}S]Thioacetic acid (ethanethiolic acid) (approx. 1·52 g., 20 m-moles, 71 mC; obtained from the Radiochemical Centre, Amersham) was distilled in high vacuum (0·001 mm. Hg) into a thick-walled Carius tube, which contained α-acetamidoacrylic acid (5·18 g., 40 m-moles; Bergmann & Grafe, 1930), cooled in liquid air (cf. Behringer, 1948). The tube was sealed and heated at 100° for 2 hr. and 120° for 1·5 hr. At the end of the reaction any volatile residue was removed by vacuum distillation at 0·001 mm. Hg and 100° into a trap cooled in liquid air. The solid product was repeatedly extracted with dry, boiling $CHCl_3$ (8 × 25 ml.), the $CHCl_3$ being removed through a filter stick and collected in a receiver to which two alkaline permanganate traps were attached in order to absorb any volatile material containing ^{35}S. The $CHCl_3$ extracts were evaporated in a stream of air to exactly 100 ml. To 5 ml. of this solution light petroleum (b.p. 60–80°) was added and the crystals formed after 48 hr. at 0° were filtered off. The material was recrystallized from ethyl acetate (yield, 169·1 mg.) and assayed for radioactivity after dilution with non-isotopic diacetyl-DL-cysteine and recrystallization from ethyl acetate to constant radioactivity. The calculated specific radioactivity of the undiluted material was 17·4 μC/mg.

DL-[^{35}S]*Cystine.* The main portion of the above $CHCl_3$ solution (95 ml. containing 55·8 mC, as calculated by assay of the diluted material) was evaporated to 30 ml. Light petroleum (10 ml.; b.p. 60–80°) was added slowly, and after 72 hr. the supernatant liquid was removed with a filter stick. After drying in a stream of air, the product was refluxed for 3 hr. with 40 ml. of a mixture of 4 N-HCl and formic acid (1:1, v/v). The acid was removed by repeated evaporation *in vacuo* at 100° and the solid residue dissolved in water (approx. 30 ml.). The pH was adjusted to 8 with conc. ammonia (sp.gr. 0·88), 2 drops of 1% (w/v) aqueous $FeCl_3$ were added and a slow stream of air was passed through the solution for 8 hr. Acetic acid was then added to pH 4·5 and after 72 hr. at 4° the pale-buff solid was collected. It was purified by dissolving in 2 N-HCl (10 ml.), treating with charcoal and neutralizing the filtrate to pH 4·5 with 6 N aqueous NH_3 and 3 N sodium acetate. The white ppt. of DL-[^{35}S]cystine was centrifuged after 72 hr. at 4°, washed with water (2 × 5 ml.), ethanol (2 × 10 ml.) and ether (20 ml.) (yield: 0·98 g., 28·8 mC). The purity of this substance was checked by paper chromatography after conversion into cysteic acid, which showed the presence of a single radioactive peak coinciding with the ninhydrin-positive spot.

Resolution of the isotopically labelled cystines

The separation of the salts of a racemic acid with an optically active base by crystallization is usually incomplete, but the more insoluble diastereoisomer can be obtained in reasonable yield and purity by repeated recrystallization. The mother liquors then contain all the more soluble salt contaminated with a small amount of the insoluble salt. When the racemic compound is isotopically labelled, it is possible to replace the unwanted labelled diastereoisomer in the mother liquors by its non-isotopic counterpart by crystallization of the mixture after addition of a large excess of the required unlabelled diastereoisomer. By this method the total amount of the contaminating diastereoisomer in solution remains unchanged, but the amount of isotope which it contains can be reduced to any desired extent by repeating the above procedure. Thus, if M is the mass of labelled contaminant of isotope content x originally present and M' is the mass of unlabelled diastereoisomer added each time, then the isotope content, y, of the contaminant remaining after n crystallizations from a constant volume is given by the formula, $y = x[M/(M+M')]^n$.

Since x and y can be measured and M' is known, it is possible to use this equation, when $n = 1$, to calculate the mass, M, of contaminant originally present, i.e. $M = M'y/(x-y)$.

This method, which has been used for the resolution of labelled leucine (Schoenheimer, Ratner & Rittenberg, 1939), was applied to the resolution of cystine labelled with ^{14}C, ^{15}N or ^{35}S described below.

Resolution of [β-^{14}C]*cystine.* *S*-Benzyl-*N*-formyl-DL-[β-^{14}C]cystine (2·71 g., 63·1 μC) and anhydrous brucine (4·4 g.) were dissolved in *n*-butanol (20 ml.) at 70°. A small amount of insoluble residue was filtered off and discarded after washing with butanol (2 × 2·5 ml.). The crystals which had formed in the filtrate (25 ml.) after 72 hr. at 19° were isolated and the mother liquors kept for a further 72 hr. at 0°. The small second crop was combined with the first and recrystallized four times from butanol (20 ml.). All mother liquors except that from the final recrystallization were combined and retained. The yield of butanol-insoluble material, i.e. the brucine salt of *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine, was 2·93 g. (26·4 μC; $[\alpha]_D^{19} = -24{\cdot}5°$ in water (*c*, 0·92); cf. Wood & du Vigneaud, 1939*b*). The conversion of this substance into D-[β-^{14}C]cystine was carried out essentially as described by Wood & du Vigneaud (1939*b*). The cystine thus obtained was dissolved in 2 N-HCl (3 ml.) and the solution was treated with charcoal (10 mg.), which was filtered off and washed with 2 N-HCl (2 × 1 ml.). The filtrate and washings were combined, 2 N aqueous NH_3 was added to pH 4·5 and the precipitated cystine was washed with water (2 ml.). Yield of D-[β-^{14}C]-cystine, 281 mg., 13·2 μC, $[\alpha]_D^{21} = +217{\cdot}3 \pm 1°$ in N-HCl (*c*, 0·549).

The combined mother liquors (approx. 80 ml.) containing the 'butanol-soluble' fraction, i.e. the brucine salt of *S*-benzyl-*N*-formyl-L-[β-^{14}C]cysteine contaminated with a small amount of the corresponding derivative of the D isomer, were evaporated to 25 ml. in a stream of air at 70°. The solution remained clear on keeping for 24 hr. at 0°. Non-isotopic *S*-benzyl-*N*-formyl-D-cysteine brucine salt (817·4 mg.) was then dissolved in the above solution by warming. After 72 hr. at 0°, the crystals were filtered off and recrystallized from butanol to constant radioactivity. The above procedure was repeated twice, using 761·5 and 892·2 mg. of non-isotopic *S*-benzyl-*N*-formyl-D-cysteine brucine salt. The radioactivity of the *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine present as a contaminant of the corresponding derivative of the L-[β-^{14}C]cysteine in the butanol solution was thus reduced successively as shown in Table 1. The butanol solution remaining after the third dilution with

Table 1. *Purification of* S-*benzyl*-N-*formyl*-L-[β-^{14}C]*cysteine brucine salt by repeated dilution of the corresponding* D-[β-^{14}C]*cysteine derivative with non-isotopic carrier*

S-Benzyl-*N*-formyl-D-cysteine brucine salt was added to dilute the radioactivity of the corresponding ^{14}C-labelled compound, most of which was then removed by crystallization from *n*-butanol (25 ml.). By repeating this procedure, the radioactivity of the *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine brucine salt remaining in solution after the third crystallization was only 5% of the original and 0·1% of that of the corresponding derivative of L-[β-^{14}C]cysteine. Details of the experimental procedure and the method for calculating the results are given in the text.

Amount of unlabelled *S*-benzyl-*N*-formyl-D-cysteine brucine salt added (g.) M'	Specific radioactivity of the D-[β-^{14}C]-cysteine derivative in solution before addition of unlabelled carrier (M') (μC/g.) x	Specific radioactivity of the D-[β-^{14}C]-cysteine derivative isolated by crystallization after addition of unlabelled carrier (μC/g.) y	Amount of brucine salt of D-[β-^{14}C]-cysteine derivative in solution (g.) $M=\frac{M'y}{x-y}$	Total radioactivity associated with the D-[β-^{14}C]cysteine derivative (M) in solution (μC) My
0·8174	9·00	2·17	0·260	0·564
0·7615	2·17	0·507	0·227	0·118
0·8922	0·507	0·114	0·260	0·030

Table 2. *Purification of* N, S-*diacetyl*-D-[^{35}S] *cysteine brucine salt by repeated dilution of the corresponding* L-[^{35}S]*cysteine derivative with non-isotopic carrier*

Experimental details, calculation of the results and definition of the symbols are given in the text. A similar experiment with *S*-benzyl-*N*-formyl-L-[β-^{14}C]cysteine is described in Table 1.

Amount of unlabelled *N*, *S*-diacetyl-L-cysteine brucine salt added (g.) M'	Volume of butanol solution (ml.)	Specific radioactivity of the L-[^{35}S]cysteine derivative in solution before addition of unlabelled carrier (M') (μC/g.) x	Specific radioactivity of the L-[^{35}S]cysteine derivative isolated by crystallization after addition of unlabelled carrier (μC/g.) y	Amount of L-[^{35}S]cysteine derivative in solution (g.) $M=\frac{M'y}{x-y}$	Total radioactivity associated with the L-[^{35}S]cysteine derivative (M) in solution (μC) My
1·468	25	230	48	0·387	18·6
0·528	25	48	20	0·377	7·5
0·689	20	20	5·5	0·261	1·4

non-isotopic brucine salt was evaporated to dryness in a stream of air at 70° and finally *in vacuo*. The yellow, glass-like residue (3·41 g.) contained 258 mg. of the brucine salt of *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine (0·029 μc, Table 1) and hence the amount of brucine salt of the L-[β-^{14}C]cysteine derivative was 3·15 g. (28·3 μC, calculated from the specific radioactivity of this salt which must be identical with that of the original pure brucine salt of the D-[β-^{14}C]cysteine derivative, i.e. 9·0 μc/g., cf. Table 1). The contamination of the *S*-benzyl-*N*-formyl-L-[β-^{14}C]cysteine brucine salt by radioactivity due to corresponding D-[β-^{14}C]cysteine derivative was therefore approx. 0·1%.

L-[β-^{14}C]Cystine was isolated from the above derivative as previously described for the D enantiomorph. Yield: 310 mg., 13·2 μc, $[\alpha]_D^{21} = -161{\cdot}0 \pm 0{\cdot}70°$ in N-HCl (*c*, 0·7). The optical rotation indicated that this material was a mixture containing 87% L- and 13% D-cystine. However, from the composition of the brucine salt (see above and Table 1) the amount of D-cystine present was expected to be only 7·6%. The difference between the radioactivity of this substance and that of the pure D-[β-^{14}C]cystine was 8·4%, which agrees well with the latter figure, since the D-cystine in the mixture has an almost negligible radioactivity. No definite explanation for the small but significant discrepancy between the optical and isotopic measurements can at present be put forward, but it is possible that a certain amount of racemization occurred during the hydrolysis of the *S*-benzyl-*N*-formyl-cysteine. The presence of occluded salt, which seemed at first another possible explanation, was excluded by X-ray powder photographs. Furthermore, such an impurity would decrease the specific radioactivity as well as the optical rotation of the cystine.

Resolution of [^{15}N]*cystine*. *S*-Benzyl-*N*-formyl-DL-[^{15}N]-cysteine was resolved exactly as previously described for [β-^{14}C]cystine. The D-[^{15}N]cystine contained 14·2 atom % ^{15}N excess and had $[\alpha]_D^{21} = +218{\cdot}4 \pm 1°$ in N-HCl (*c*, 0·568). The 'L-cystine' fraction had $[\alpha]_D^{21} = -148{\cdot}1 \pm 0{\cdot}7°$ in N-HCl (*c*, 0·562). It was calculated to contain 16% of the D enantiomorph by measurement of the optical rotation or 13% by the isotope dilution procedure given above. The isotope content of this fraction was 12·3 atom % ^{15}N excess by direct analysis and the composition of the mixture was 0·083 g. D-[^{15}N]cystine (0·96 atom % excess) and 0·6226 g. L-[^{15}N]cystine (14·2 atom % excess).

Resolution of [^{35}S]*cystine*. In trial experiments with non-isotopic material it was found that a solution of *N*, *S*-diacetyl-DL-cysteine brucine salts in *n*-butanol (0·1 g. *N*, *S*-diacetyl-DL-cysteine/ml.) on cooling gave the brucine salt of *N*, *S*-diacetyl-L-cysteine in 95% yield. After recrystallization from *n*-butanol to constant rotation, this salt

had m.p. 171–172°, $[\alpha]_D^{22} = -28{\cdot}4°$ in water (*c*, 1·974). Its solubility in butanol was 0·460 g./100 ml. at 0°. Conversion into the free amino acid afforded pure L-cystine, $[\alpha]_D^{23} = -223{\cdot}1 \pm 1°$ in N-HCl (*c*, 0·674).

N, *S*-Diacetyl-DL-[^{35}S]cysteine (2·50 g., 1·57 mc) and anhydrous brucine (4·81 g.) were dissolved in warm butanol (25 ml.). After 72 hr. at 4°, the crystalline solid was separated and recrystallized twice from butanol (12 ml.). The combined mother liquors were concentrated to 25 ml. in a stream of air at 70° and kept at 0° for 104 hr., giving a small amount of crystalline material which was added to the first crop. After three recrystallizations from butanol (12 ml.), the yield of *N*, *S*-diacetyl-L-[^{35}S]cysteine brucine salt was 2·86 g. (0·635 mc). The salt was dissolved in N aqueous NH_3 (30 ml.) and the precipitated brucine washed with 0·5N aqueous NH_3 (3 × 10 ml.). The solution and washings were evaporated *in vacuo* at 50° to remove the NH_3 and the crude *N*, *S*-diacetyl-L-[^{35}S]cysteine was hydrolysed to cystine, which was purified as described above. Yield: 353 mg., 288 μc; $[\alpha]_D^{20} = -221 \pm 1°$ in N-HCl (*c*, 0·340).

To the mother liquors remaining after removal of the second crop of the *N*, *S*-diacetyl-L-[^{35}S]cysteine brucine salt were added successively three amounts of the corresponding non-isotopic compound (Table 2), essentially as described for the resolution of the [β-^{14}C]cystine. The butanol solution remaining after the third dilution with non-isotopic brucine salt was evaporated to dryness in a stream of air at 70° and finally *in vacuo*, giving a glass-like yellow gum (3·272 g.). This material was a mixture of the brucine salts of *N*, *S*-diacetyl-L-[^{35}S]cysteine (261 mg., 1·4 μc) and *N*, *S*-diacetyl-D-[^{35}S]cysteine (3·011 g., 692·5 μc). It was converted by methods already described into 'D-[^{35}S]cystine' (424·1 mg., 389 μc), which had $[\alpha]_D^{21} = +167{\cdot}8 \pm 0{\cdot}7°$ in N-HCl (*c*, 0·070) and thus contained 11 % of the L enantiomorph. This value is in fair agreement with that of 8 % calculated by the dilution technique. It can also be calculated that only approx. 0·09 % of the total radioactivity of this material was associated with the L enantiomorph.

RESULTS

The present experiments (Table 3), as well as the results previously obtained with small-scale fermentations (Arnstein & Grant, 1954), show that L-cystine is used much more efficiently for penicillin biosynthesis than the D enantiomorph. As in the earlier work, degradation of the labelled penicillin (Table 4) showed that the β-carbon atom of cystine was incorporated exclusively into $C_{(5)}$ of penicillin. It was also found that the penicillamine moiety contained practically no isotopic nitrogen but that all the ^{15}N was incorporated into the side-chain

Table 3. *Conversion of* L- *and* D-*cystine, labelled with* ^{14}C, ^{15}N *and* ^{35}S, *into penicillin and mycelial cystine*

Solutions of L-[β-^{14}C, ^{15}N, ^{35}S]cystine (total 267·5 mg.; 4·22 μc ^{14}C, 3·11 atom % excess ^{15}N, 8·37 μc ^{35}S) or D-[β-^{14}C, ^{15}N, ^{35}S]cystine (total 267·5 mg.; 4·29 μc ^{14}C, 4·10 atom % excess ^{15}N, 9·56 μc ^{35}S) in N-HCl (about 3 ml.) were sterilized by Seitz-filtration and the filter was washed with N-HCl to give a final vol. of filtrate + washings of 5 ml. 1 ml. portions were added every 24 hr. to identical fermentations, the first addition being made at zero time, the last at 96 hr. after inoculation of the medium. At the end of the fermentation, i.e. 22 hr. after the final addition of cystine, the yields of sodium benzylpenicillin, calculated by bioassay, were 239 mg. and 120 mg., respectively. The amount of radioactivity incorporated into penicillin from the labelled L-cystine was 0·315 μc ^{14}C (7·5 %) and 0·751 μc ^{35}S (9·0 %), that from the D-cystine was 0·034 μc ^{14}C (0·79 %) and 0·15 μc ^{35}S (1·6 %). Penicillin was isolated from the broth as described in the text. The potencies of the penicillin *N*-ethylpiperidine salt in the L- and D-cystine experiments were respectively 1·037 and 1·019 times that of the corresponding derivative of International Standard penicillin (1670 i.u./mg.), the fiducial limits of the assay being ±1·4 % ($P < 0{\cdot}05$). The standard error of all radioactivity assays was less than 5 %. ^{15}N assays were accurate to ±0·003 atom % excess. The ^{15}N results in columns 3 and 4 were calculated from assays of material which had been diluted with carrier and are therefore less accurate, as indicated. The figures in parentheses in column 6 are the range of the dilution of ^{15}N incorporation into mycelial cystine (*A*/*C*). Other details are given in the Experimental section.

	Specific radioactivity (μc/m-mole) or atom % excess ^{15}N			Dilution of isotope	
Isotope	Cystine* added to fermentation (*A*)	Penicillin isolated (*B*)	Cysteic acid derived from mycelial cystine (*C*)	Penicillin (*A*/*B*)	Mycelial cystine (*A*/*C*)
	L-Cystine experiment				
^{14}C	1·91	0·47	0·35†	4·1	5·5
^{15}N	3·11 ± 0·003	0·83‡ ± 0·01	0·52 ± 0·09	3·75 ± 0·05	6·0 (5·1–7·25)
^{35}S	3·79	1·12	0·77	3·3	4·9
	D-Cystine experiment				
^{14}C	1·94	0·10	0·08†	19·4	24·2
^{15}N	4·09 ± 0·003	0·16‡ ± 0·01	0·10 ± 0·085	25·6 ± 1·6	40·9 (22·1–273)
^{35}S	4·33	0·44	0·285	9·9	15·2

* The specific radioactivity of this substance is given as μc/m-mole cysteine. Where necessary, the specific radioactivity and ^{15}N content of the cystine used are corrected for the presence of the optical enantiomorph, which was practically free of isotopic tracer (see Experimental section).

† Calculated by difference from the radioactivity of cysteic acid and that of the cysteic acid sulphur after conversion into benzidine sulphate.

‡ Calculated for one N atom being derived from the precursor (see degradation, Table 4).

Table 4. *Degradation of labelled penicillin isolated from fermentations containing* L- *or* D-[β-^{14}C, ^{15}N, ^{35}S]*cystine*

Details of the fermentations, assay and degradation are given in Table 3 and in the Experimental section. In order to compare the ^{15}N content of different compounds, the ^{15}N results are given on the basis of all the isotope being located in one N atom, i.e. the atom % ^{15}N excess in the compounds was multiplied by the number of nitrogen atoms. For numbering of the penicillin molecule see Arnstein & Grant (1954).

Compound	Atom number in thiazolidine and β-lactam rings of penicillin	Specific radioactivity (μc/m-mole) or atom % excess ^{15}N: L-Cystine expt. ^{14}C, ^{35}S	L-Cystine expt. ^{15}N	D-Cystine expt. ^{14}C, ^{35}S	D-Cystine expt. ^{15}N
Benzylpenicillin *N*-ethylpiperidine salt	All	1·59	0·83±0·01	0·54	0·16±0·01
$BaCO_3$ from acid-labile CO_2	7	Inactive	—	Inactive	—
Isopropylidenepenicillamine HCl	(1+2+3)	1·13	0·04	0·44	None
Benzidine sulphate	1	1·11	—	0·43	—
Benzylpenilloaldehyde 2:4-dinitrophenylhydrazone	(5+6)	0·47	0·80±0·015	0·12	0·165±0·015
2-Naphthalenesulphonylglycine	(5+6)	0·44	0·80±0·003	0·09	Not determined
$BaCO_3$ from glycine carboxyl carbon atom	5	0·47	—	0·10	—
Formaldehyde dimedon from glycine α-carbon atom	6	Inactive	—	Inactive	—

Table 5. *Conversion of* L-[β-^{14}C, ^{35}S]*cystine into penicillin and into mycelial cystine and serine*

The experimental conditions were similar to those described in Table 3, but less labelled cystine was used. L-[β-^{14}C, ^{35}S]-Cystine (34·6 mg., containing 1·7 mg. almost completely non-radioactive D-cystine, present in the ^{14}C-labelled cystine as explained in the Experimental section; 0·546 μc ^{14}C, 0·507 μc ^{35}S) was divided into six equal portions which were added every 24 hr. starting at zero time. After 139 hr. the fermentation medium contained 345000 i.u. corresponding to 202 mg. sodium benzylpenicillin (0·0822 μc ^{14}C, 0·0817 μc ^{35}S). Penicillin was isolated in the usual way. Cystine was isolated from the mycelium as cysteic acid and the radioactivity in the β-carbon atom of the mycelial serine was determined as described in the Experimental section. The standard error of all radioactivity determinations was less than 5%.

Isotope	Specific radioactivity (μc/m-mole): Cystine added to fermentation* (*A*)	Penicillin isolated (*B*)	Cysteic acid derived from mycelial cystine (*C*)	Formaldehyde dimedon from β-carbon atom of mycelial serine (*D*)	Dilution of isotope: Penicillin (*A*/*B*)	Mycelial cystine (*A*/*C*)	β-Carbon atom of mycelial serine (*A*/*D*)
^{14}C	2·01	0·145†	0·087‡	0·0035	14·3	23·0	572
^{35}S	1·87	0·144	0·093	—	13·0	20·1	—

* Calculated as μc/m-mole cysteine.

† Calculated by difference from the molar radioactivity of penicillin *N*-ethylpiperidine salt and that of benzidine sulphate (Table 6).

‡ Calculated by difference from the radioactivity of cysteic acid and that of the cysteic acid sulphur after conversion into benzidine sulphate.

Table 6. *Degradation of labelled penicillin isolated from a fermentation containing* L-[β-^{14}C, ^{35}S]*cystine*

Details of the fermentation and degradation are given in Table 5 and in the Experimental section. The numbering of the penicillin atoms is as in Table 4. For the degradation 29·0 mg. of the radioactive penicillin *N*-ethylpiperidine salt was diluted with 170·0 mg. non-radioactive carrier. The figures in parentheses in column 3 are the calculated radioactivities corresponding to the undiluted penicillin.

Compound	Atom number in thiazolidine and β-lactam rings of penicillin	Specific radioactivity (μc/m-mole)
Penicillin *N*-ethylpiperidine salt	All	0·0416 (0·286)
Isopropylidenepenicillamine HCl	(1+2+3)	0·0220 (0·151)
Benzidine sulphate	1	0·0206 (0·141)
Benzylpenilloaldehyde 2:4-dinitrophenylhydrazone	(5+6)	0·0199 (0·137)

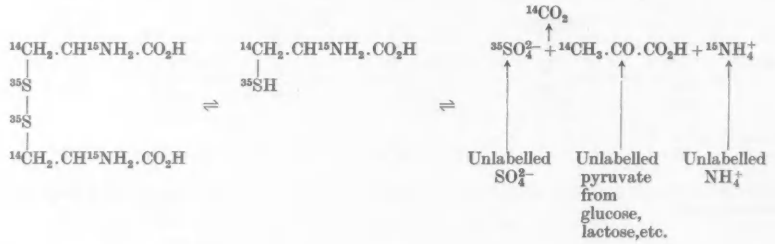
amide group. As expected, the penicillin also contained radioactive sulphur. In the experiment with L-cystine, labelled with isotopic carbon, nitrogen and sulphur (Table 3), the calculated ratios of isotopes ($^{35}S:^{15}N:^{14}C$) in the precursor and in penicillin were similar, being 1·99:1·63:1 and 2·49:1·77:1, respectively. The $^{15}N:^{14}C$ ratios show excellent agreement, but the sulphur of the added cystine appeared to be converted into penicillin with a significantly lower dilution than the other isotopes, resulting in an unexpectedly high $^{35}S:^{14}C$ ratio. In this experiment, the cystine isolated as cysteic acid from the mycelium at the end of the fermentation was found to contain ^{35}S, ^{15}N and ^{14}C in the ratio 2·43:1·64:1, which is essentially the same as that in the penicillin. However, the incorporation of all three isotopes into the mycelial cystine was significantly less than into penicillin and a similar result was obtained in the experiments with similarly labelled D-cystine and also with L-[β-^{14}C, ^{35}S]cystine added to a fermentation in much smaller amount (Table 5). In the latter experiment (Tables 5 and 6) the $^{35}S:^{14}C$ isotope ratios in the precursor, in cystine from the mycelium and in the penicillin showed a somewhat better agreement (0·93, 1·07 and 0·99, respectively), although the sulphur isotope was still to some extent preferentially utilized. The radioactivity of the β-carbon atom of the serine present in the mycelium was also determined and found to be only about 4 % of the radioactivity due to ^{14}C in the cystine from the same source and 2·4 % of that of the $C_{(5)}$ of the isolated penicillin.

DISCUSSION

The efficient utilization of L-cystine, labelled with ^{14}C in the β-position, for penicillin biosynthesis, which was shown in the preceding paper (Arnstein & Grant, 1954), suggested that this amino acid may be an immediate precursor of penicillin. Since penicillin formation in this case would involve only reactions in which the cystine molecule remains intact apart from a reduction to cysteine, L-[β-^{14}C, ^{15}N, ^{35}S]cystine should give rise to penicillin in which the isotopic carbon, nitrogen and sulphur are located at carbon-atom 5, the nitrogen of the side-chain amide group and the sulphur atom, respectively. Furthermore, the three isotopes should be incorporated into these positions to an equal extent.

In the present work, degradation of the penicillin obtained in such an experiment showed that the isotopes were indeed located in the expected positions. However, only the carbon and nitrogen isotopes were incorporated with similar dilutions. In all experiments, a small but significant excess of radioactive sulphur relative to the other isotopes was found in the penicillin. The unexpectedly high utilization of cystine sulphur may be due to the endogenous synthesis of cystine containing radioactive sulphur but relatively little isotopic carbon or nitrogen.

One mechanism by which cystine labelled originally with isotopic sulphur, carbon and nitrogen might be converted into cystine containing relatively more isotopic sulphur than carbon and nitrogen is illustrated below.



In the experiment with labelled D-cystine (Table 3), the $^{35}S:^{15}N:^{14}C$ ratios in the added cystine, in the penicillin and in the mycelial cystine were found to be 2·24:2·11:1, 4·4:1·6:1 and 3·56:1·25:1, respectively. It appears that in this case the isotopic sulphur is converted into penicillin and into the L-cystine of the mycelium with about twice the efficiency of the utilization of ^{14}C. The change in the $^{15}N:^{14}C$ ratio also indicates that relative to the carbon some of the nitrogen is lost during the conversion of the D-cystine into penicillin or into the mycelial cystine.

It is known (cf. Arnstein & Grant, 1954) that cystine is oxidized to carbon dioxide during the fermentation and it is likely that in this reaction the sulphur of cystine is converted into sulphate, possibly by a pathway similar to that known to occur in animal tissue (Medes & Floyd, 1942; cf. Kearney & Singer, 1953). Sulphate is used efficiently for penicillin biosynthesis (Lester Smith & Hockenhull, 1952) and is, of course, also a precursor of cystine, since it can serve as the sole sulphur source in fermentation media. By analogy with the metabolism of cystine in animal tissues, pyruvate has been assumed to be

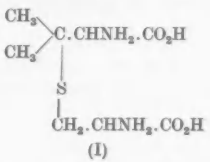
the primary sulphur-free metabolic product, but such an assumption is not essential to the present discussion. The above sequence of reactions leading to the degradation and resynthesis of cystine would be expected to result in a greater dilution of the carbon and nitrogen isotopes than of the sulphur isotope, because much more unlabelled carbon (as glucose, lactose, etc.) and nitrogen (as ammonia) than sulphur (as sulphate) are present in the fermentation medium. Including the relatively small amount of cystine added, the fermentation solution contains approximately 16 g. C, 2 g. N and 0·2 g. S/l.

In support of the above hypothesis that the unexpectedly high incorporation of ^{35}S into penicillin is due to re-incorporation of radioactive sulphate during cystine synthesis, it was found that the cystine present in the mycelium at the end of the fermentation also contained a definite excess of radioactive sulphur, compared with the carbon and nitrogen isotopes. In fact, the $^{35}S:^{15}N:^{14}C$ ratios in the mycelial cystine and in the relevant atoms of penicillin were practically the same. Moreover, a decrease in the quantity of labelled cystine added from 1·0 to 0·14 m-mole decreased the extent of preferential utilization of the sulphur isotope. It is likely that in this case less of the labelled cystine was oxidized to sulphate. It is also of interest that, at the lower level, cystine was used for penicillin synthesis with about twice the efficiency, 15% of the added ^{14}C being converted into penicillin compared with 7·5% at the higher level.

In the preceding paper (Arnstein & Grant, 1954) it was assumed that the lower specific radioactivity of the penicillin compared with the cystine added to the fermentation was due to the dilution of the labelled amino acid by unlabelled cystine synthesized endogenously by the mould. This interpretation is supported by the present results, which show that the isotope contents of the cystine incorporated into the mycelial protein and of the penicillin are of the same order of magnitude. On the assumption that the added labelled cystine mixes freely with the endogenously produced cystine, it may be calculated that about 2–5 m-moles of cystine are synthesized during the course of a 1 l. fermentation. One explanation of the differences in the absolute values of the isotope contents of the mycelial cystine and of the penicillin, which range from 25 to 50%, may be that protein synthesis occurs mainly during a stage of the fermentation in which the amount of endogenous cystine synthesized is somewhat larger than that produced during the period of penicillin biosynthesis. Most of the cystine incorporated into the mycelial protein would thus have a slightly lower radioactivity than that used for penicillin formation. This interpretation is in agreement with the observation that mycelium formation by *Penicillium chrysogenum* WIS 48–701 on a corn-steep medium is essentially complete before the beginning of penicillin production (cf. Anderson *et al.* 1953).

The poor utilization of D-cystine for mycelial protein and penicillin biosynthesis confirms the earlier work with micro-fermentations, in which D-cystine was found to be a less efficient penicillin precursor than the L enantiomorph. It is probable that D-cystine is converted into L-cystine before it is used for penicillin formation, since the relative incorporation of both enantiomorphs into penicillin and into the cystine of the mycelial protein were very similar. The inversion of D amino acids is well known, especially in animals, which can use the D enantiomorphs of certain essential amino acids for growth. In experiments with ^{15}N-labelled leucine and γ-phenyl-α-aminobutyric acid it has been demonstrated that rats are able to convert the D into the corresponding L amino acids. In this reaction most of the labelled amino nitrogen was lost, presumably because the corresponding keto acids were formed as intermediates (Ratner, Schoenheimer & Rittenberg, 1940; du Vigneaud *et al.* 1939). It is therefore of interest that in the fermentation the conversion of D-cystine into L-cystine takes place largely without loss of the labelled amino group. It is possible that in this case an imino acid is an intermediate or, alternatively, the ammonia formed by deamination may be only in slow equilibrium with the unlabelled ammonia of the fermentation medium. This would result in reamination of the intermediate keto acid with ammonia of substantially the same isotope content as that of the D amino acid.

While the present experiments with labelled cystine indicate that this amino acid is an important penicillin precursor they provide relatively little information about the individual reactions involved in the conversion of cystine into penicillin. The first step consists presumably of a reduction to cysteine, which may then condense with valine or a close derivative of this amino acid. Alternatively, cysteine (or cystine) may first react with phenylacetic acid to give phenylacetylcysteine as an intermediate. Condensation of cysteine and valine may lead either to a dipeptide or to $\beta\beta$-dimethyllanthionine (I). Since (I) could possibly also arise from penicillamine and serine by



(I)

analogy with the condensation of serine and homocysteine to cystathionine, it was important to

compare cystine and serine as possible intermediates in penicillin biosynthesis. Earlier experiments (Arnstein & Grant, 1954) had shown that labelled serine was converted into penicillin, but with a somewhat higher dilution than cystine. More conclusive evidence that serine is not an intermediate in the synthesis of penicillin from cystine is provided by the observation that the serine present in the mycelial protein of a fermentation to which ^{14}C-labelled cystine had been added was only very weakly radioactive compared with the cystine from the same source or with the isolated penicillin. It is therefore likely that serine is used for penicillin biosynthesis only after conversion into cystine by a substantially irreversible reaction.

The complete elucidation of the mechanism by which cysteine is converted into penicillin must await further experiments. It is of interest, however, that the participation of penicillamine appears to be excluded by the present work, which suggests that the penicillamine moiety of penicillin is derived from valine. Penicillamine should therefore no longer be regarded as a naturally occurring amino acid but solely as an artifact produced by the chemical degradation of penicillin.

SUMMARY

1. The preparation of L- and D-cystine, labelled with ^{14}C in the β-position, ^{15}N or ^{35}S, is described. By appropriate dilution with unlabelled carrier, each optically active form was obtained practically free from labelled enantiomorph.

2. The conversion of L- and D-[β-^{14}C, ^{15}N, ^{35}S]- and L-[β-^{14}C, ^{35}S]-cystine into penicillin and into the cystine of the mycelial protein has been studied.

3. Penicillin and cysteic acid from the mycelial cystine were chemically degraded in order to ascertain the distribution of the isotopes.

4. The labelled L-cystine was a more efficient precursor of penicillin and of mycelial cystine than the D enantiomorph.

5. The ratios of the ^{14}C, ^{15}N and ^{35}S isotopes in the mycelial cystine and in the labelled atoms of penicillin were identical and similar to that of the L-cystine which had been added to the fermentation.

6. It is concluded that cystine is a direct precursor of penicillin, probably after reduction to cysteine. Its role in penicillin biosynthesis is discussed.

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The Tryptophanase-Tryptophan Reaction. The Nature of the Enzyme-Coenzyme-Substrate Complex

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Baker & Happold (1940) first suggested a $C_{(\alpha)}$–$C_{(\beta)}$ fission in the tryptophan molecule as the essential process catalysed by tryptophanase. They showed that a free carboxyl group and an unsubstituted α-amino group were essential in the substrate, and since β-3-indolylglycine was not acted upon by tryptophanase they felt that the mechanism might involve oxidation at the β-carbon atom of the alanine side chain. Baker, Happold & Walker (1946) showed that an unsubstituted imino group was necessary in the indole nucleus since β-1-methyl-3-indolylalanine was not a substrate. These authors believed that the 3-carbon fragment which was separated from the indole nucleus was alanine, but Wood, Gunsalus & Umbreit (1947) showed that the reaction could be represented as

$$\text{tryptophan} \xrightarrow{\text{tryptophanase}} \text{indole} + \text{pyruvic acid} + \text{ammonia}$$

and that pyridoxal phosphate was the coenzyme for this system. These results were confirmed by Dawes & Happold (1949). The present work extends the previous findings of Baker *et al.* (1940, 1946) by determining the effect of the enzyme on an additional number of structurally related compounds and also the inhibitory effect of these compounds on the tryptophan-tryptophanase reaction. We feel that it is now possible to postulate that the system is a ternary complex of enzyme, coenzyme and substrate, and to suggest the most likely points of attachment between the three components.

MATERIALS AND METHODS

Cell-free extracts of the enzyme were obtained from cells of *Escherichia coli* by the method of Dawes & Happold (1949). Indole formed in the enzyme activity tests was determined by the method of Happold & Hoyle (1934). The rosindole colour formed by the addition of Ehrlich's reagent was measured in a Unicam D.G. Spectrophotometer at a wavelength of 5600 Å and is stable over a period of 15 min. Duplicate standard solutions can be estimated to within 1 μg. under the conditions used in the experiments.

Pyruvate was determined by the method of Friedemann & Haugen (1943), using the toluene-extraction procedure. The colours formed were measured in the spectrophotometer at a wavelength of 5200 Å. The standard colours for comparison were obtained from lithium pyruvate prepared by the method of Wendl (1931). Ammonia was determined by the microdiffusion method of Conway & O'Malley (1942).

Initial rate of enzyme action. This was determined by mixing 3 ml. of 0·0133 M phosphate buffer, pH 7·8, containing 10 μg. pyridoxal phosphate, and 1 ml. of enzyme solution and incubating for 10 min. at 37°. The substrate solution (1 ml. of 0·0196 M L-tryptophan which had been incubated separately) was then added over a period of 5 sec. and the reaction allowed to proceed for the required length of time; this was usually 5 min. but in the early experiments periods of up to 30 min. were employed. The reaction was stopped by the addition of 5 ml. of 10% (w/v) trichloroacetic acid (TCA) and the precipitated protein removed by centrifuging or filtration. The indole, pyruvate and ammonia were then determined on samples of the supernatant. The figure given is the average of two determinations. The initial rate was calculated as μg. indole formed/ml. TCA sample/min., measured over the first 5 min. of the reaction time.

EXPERIMENTAL AND RESULTS

Rate of indole production

The rate of enzymic production of indole from tryptophan is shown in Fig. 1, in which three different cell-free enzyme extracts from *Esch. coli* have been used; where indole production was approximately 20 μg. in 5 min., the rate of reaction

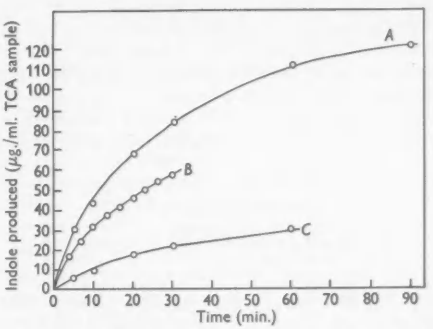

Fig. 1. Enzymic rate of indole production. *A*, *B* and *C* represent different enzyme preparations. Systems contained 1 ml. of enzyme, 1 ml. of L-tryptophan (0·0196 M) and 3 ml. of 0·0133 M Na_2HPO_4, KH_2PO buffer, pH 7·8.

was constant, so 5 min. was chosen as the time for the normal activity test. The rate of indole production after this point was not constant and the curve for enzyme solution *A* shows that 30 μg. indole were produced in the first 5 min., but only 9 μg. between 60 and 90 min. The decrease in rate in this experiment could not be accounted for solely in terms of decreasing substrate concentration.

Action of tryptophanase on derivatives of indole and indene

Activity tests were carried out by the above method using the following indole derivatives as substrates: 3-indolylacetic acid, β-3-indolylpropionic acid, β-3-indolylbutyric acid and β-3-indolylethylamine (all 2·12 mM), DL-α-amino-β-3-indolylbutyric acid (1·88 mM) and D-tryptophan (19·6 mM). In no case was any indole produced by the enzyme in 30 min. The inert nature of D-tryptophan had been indirectly shown by Woods (1935) for washed suspensions, by estimating the total indole produced from equimolar solutions of L- and DL-tryptophan. With the cell-free enzyme the results in Table 1 show that the rate of breakdown of solutions of L- and DL-tryptophan equimolar with respect to the L-form is the same and indicate that no inhibition occurs through the D isomer. This is studied in more detail later.

α-Amino-β-3-indenylpropionic acid (Groves & Swann, 1951) would be expected to behave similarly to tryptophan, provided the ring nitrogen atom was not essential to the reaction, producing indene, pyruvic acid and ammonia. L-Tryptophan and α-amino-β-3-indenylpropionic acid (1·96 mM) were compared as substrates in enzyme activity tests. The production of indole, ammonia and pyruvic acid from tryptophan was confirmed after 5 min. but no pyruvic acid or ammonia could be found from α-amino-β-3-indenylpropionic acid in reaction periods up to 30 hr. duration.

The inability of tryptophanase to cause any fission of the present group of compounds, and the earlier work of Baker & Happold (1940) and that of Baker *et al.* (1946) suggests strongly that the enzyme only acts on a compound containing an intact α-amino grouping, a carboxylic acid grouping and a ring nitrogen atom unsubstituted by alkyl groups. The failure to react with DL-amino-β-3-indolylbutyric acid stresses the importance of the 3-carbon side chain. The enzyme attacks only one stereoisomeric form of the substrate and requires the presence of the ring nitrogen atom for the reaction to occur. The nitrogen atom may be concerned in the combination of substrate and enzyme, in which case the indole nucleus may act as an inhibitor of the reaction. Wood *et al.* (1947) showed that added indole inhibited tryptophan breakdown, but Beerstecher & Edmonds (1951), using washed suspensions of *Esch. coli*, claimed that indole is probably required in catalytic amounts for the breakdown of tryptophan.

Table 1. *Action of tryptophanase on* L- *and* DL-*tryptophan*

	Final concentration of tryptophan (mM)					
	L	DL	L	DL	L	DL
	1·96	3·92	0·98	1·96	0·49	0·98
Time (min.)	Indole produced (μg./ml. TCA sample)					
5	17·5	18·0	14·0	13·6	8·0	7·0
10	33·4	33·6	—	—	—	—
15	40·5	42·8	—	—	—	—

Inhibition of tryptophanase by indole

We have found that the drop in the rate of indole formation by enzyme action after the first 5 min., which was not due solely to the drop in substrate concentration, was also not due to (*a*) loss of indole from the reaction vessels by volatilization or utilization, for periods up to 30 min., (*b*) enzyme denaturation during the reaction period since the activity of enzyme preparations incubated for periods up to 1 hr. before addition of substrate, did not drop, or (*c*), removal of the coenzyme from the reaction system since the initial rate did not vary with increases in the concentration of added coenzyme. Inhibition by indole seemed probable and was confirmed as follows. Indole and enzyme were incubated together for 10 min. before the addition of substrate. The concentration of L-tryptophan was 1·96 mM and that of the inhibitor, indole, 0·21 mM. The initial rate of indole production in the absence of added indole was 5·2 μg./min.; with the inhibitor present this dropped to 2·9 μg./min. The effect of varying the pre-incubation time of enzyme with indole upon the initial rate of the reactions was next tested for periods varying by 5 min. intervals from 5 to 30 min. There was no additional inhibitory effect with time after the first interval of time; equilibrium was quickly reached between the participants.

To study the type of inhibition involved, comparisons were made of the amount of inhibition when the substrate concentration was kept constant and the inhibitor concentration was varied, and vice versa. The results obtained with two different enzyme solutions are given in Table 2.

It should be noted that when indole is used as an inhibitor indole production is obtained by difference, the errors involved are increased and duplicate results vary by up to 2 μg., i.e. 0·4 μg. in the initial rate. With high substrate concentration the inhibitor concentration rapidly increases but in spite of this and the other experimental limitations the results show that at constant substrate concentra-

Table 2. *Effect of inhibitors on initial rate of reaction of tryptophanase with* L-*tryptophan*

Enzyme (1 ml.), 0·0133 M Na_2HPO_4, KH_2PO_4 buffer (2 ml.; pH 7·8) containing 10 μg. pyridoxal phosphate and inhibitor (1 ml.) were incubated together for 10 min. at 37°. L-Tryptophan (1 ml.) was then added and measurement of indole produced was made as in the Methods section. Concentrations of substrate and inhibitor are those obtaining after addition of substrate. The results were compared with those of controls containing water instead of inhibitor and the inhibition is expressed as the percentage decrease in the initial rate caused by the presence of inhibitor. Two different enzyme solutions (*a* and *b*) were used.

		L-Tryptophan in digest (mM)						
		7·0	1·96		0·98		0·49	
			a	*b*	*a*	*b*	*a*	*b*
Inhibitor	Inhibitor concn. (mM)	Inhibition (%)						
Indole	0·034	10	21	16	22	33	37	38
	0·068	32	57	33	73	51	87	94
3-Indolylacetic acid	0·068	—	0	0	—	—	—	—
	1·06	—	38	32	—	—	—	—
DL-α-Amino-β-3-indolylbutyric acid	1·88	33	—	—	—	—	66	—
β-3-Indolylpropionic acid	0·034	—	0	—	10	—	0	—
	0·068	—	0	—	5	—	0	—
	1·06	33	40	44	—	56	—	10
	2·12	54	—	59	—	73	—	91
β-3-Indolylethylamine	1·04	8	—	7	—	11	—	—
	2·07	11	—	10	—	12	—	—
D-Tryptophan	1·96	—	—	0	—	—	—	—
DL-β-1-Methyl-3-indolylalanine	3·49	—	—	0	—	—	—	—
	6·18	—	—	6	—	—	—	—

tion added indole causes inhibition. This increased inhibition is also produced at constant indole concentration by a decrease in substrate concentration. The results are consistent with competition by the inhibitor, indole, for the enzyme in place of the substrate. This presumably involves the ring nitrogen atom which is common to both structures, and in consequence other structural analogues of indole and tryptophan were investigated for the ability to inhibit tryptophan breakdown.

Structural analogues of L-*tryptophan and indole as inhibitors of tryptophan breakdown*

β-3-Indolylpropionic acid. This was tested in a similar manner to that previously described for indole. Inhibition of the reaction was found but there was no difference in the degree of inhibition when the order of mixing the participants was varied. The effects of varying the inhibitor and substrate concentrations as measured by the initial rate are also recorded in Table 2.

The results show that low concentrations of indolylpropionic acid have little effect; similar concentrations of indole produced substantial inhibition. Higher concentrations of the indole acid inhibited enzyme action. Results with other indolyl acids are also given.

β-3-Indolylethylamine. The indolylethylamine has only a slight inhibitory effect in concentrations at which the indolylpropionic acid inhibited strongly (Table 2).

D-*Tryptophan and β-1-methyl-3-indolylalanine.* As shown in Table 2, these do not inhibit tryptophanase.

Indene. This was prepared by distillation of a commercial sample (British Drug Houses Ltd.) under reduced pressure at 65°. A concentrated solution was prepared in ethanol and added to different enzyme preparations but only within a range in which the added ethanol caused no inhibition of the reaction. At 0·173 mM concentration, indene caused no decrease in the initial rate of indole formation with a substrate concentration of 0·196 mM. Indole in this concentration inhibited strongly.

α-Amino-β-3-indenylpropionic acid. This substance showed different effects as an inhibitor of the tryptophanase system according to the order in which the reactants were mixed. No, or little, inhibition was produced when the enzyme was added to a mixture of the substrate and test substance, but when the latter was pre-incubated with the enzyme, definite inhibition was found. This is shown by the results in Tables 3 and 4. α-Amino-β-3-indenylpropionic acid appears to inhibit the reaction when mixed with the enzyme and incubated for 30 min. prior to the addition of substrate. The amount of inhibition produced was small compared to that given by similar concentrations of indole acids.

Table 3. *Effect of α-amino-β-3-indenylpropionic acid on initial rate when incubated for 10 min. with substrate before addition of enzyme*

For experimental conditions see Table 2.

L-Tryptophan (mM)	α-Amino-β-3-indenylpropionic acid (mM)	Initial rate (μg. indole/ml. TCA sample/min.)
3·43	0	3·8
	2·45	3·4
1·96	0	4·0
	1·23	3·9
0·245	0	4·3
	0·49	4·2
	2·45	3·9

Table 4. *Effect of the incubation of α-amino-β-3-indenylpropionic acid with enzyme before the addition of substrate*

For experimental conditions see Table 2.

Time of pre-incubation (min.)	L-Tryptophan (mM)	
	1·96	0·49
	Initial rate (μg. indole/ml. TCA sample/min.)	
Enzyme		
5	5·1	3·3
30	5·0	3·5
Enzyme and α-amino-β-3-indenylpropionic acid ($1·23 \times 10^{-3}$ M)		
5	4·3	2·6
15	—	2·7
20	4·2	—
30	3·7	—

DISCUSSION

The previously postulated structural requirements necessary in a substrate for tryptophanase have been re-investigated using cell-free enzyme and the investigation has been extended by testing structural analogues of the substrate as inhibitors of enzyme action. In the previous and present studies, indole-3-substituted compounds were tested as substrates for the enzyme but no substance other than L-tryptophan has yet been found to yield indole by tryptophanase action. The results of the present inhibition experiments are summarized thus.

Inhibition was produced by indole, 3-indolylacetic acid, β-3-indolylpropionic acid and DL-α-amino-β-3-indolylbutyric acid.

No inhibition (or slight) was produced by indene, β-3-indolylethylamine, β-1-methyl-3-indolylalanine, D-tryptophan and α-amino-β-3-indenylpropionic acid.

The inhibition by indole was previously reported by Wood *et al.* (1947). This inhibition may account for the enzymic oxidation of tryptophan not proceeding to completion, the progressive increase in the concentration of indole halting the reaction. Of the final products of the reaction, namely, indole, pyruvic acid and ammonia, only indole inhibits, and indeed ammonium, potassium and rubidium ions increase the activity of the enzyme-coenzyme-substrate system (Happold & Struyvenberg, unpublished).

Fildes & Rydon (1947), in their studies on the tryptophan desmolase system of Tatum & Bonner (1944), suggested that a type of hydrogen bonding was formed between the ring nitrogen atom and two side chains of an enzyme molecule. Although the two enzyme systems are different, a similar suggestion might be applied to the system we have studied, although a double attachment between the ring nitrogen atom and two side chains of the enzyme molecule would not be involved.

The failure of the enzyme to react with β-1-methyl-3-indolylalanine would agree with the view that some sort of bonding occurs between the enzyme complex and the ring nitrogen atom and consideration of the structures of indole (whose inhibition of tryptophanase reaction is a function of indole and tryptophan concentrations) and indene (which does not inhibit) supports this assumption.

The failure of the enzyme to react with β-3-indolylethylamine and of this compound to inhibit indole formation by tryptophanase action supports the view that a carboxyl group is required for combination with the enzyme. β-3-Indolylpropionic acid, which possesses the carboxyl group of the usual substrate but not the α-amino group, is an inhibitor as might be expected if the acid group is involved in enzyme combination. The results show that the indole acid is not as good an inhibitor as indole itself, but since the acid possesses a side chain, a certain orientation on the surface of the enzyme would be necessary for inhibition which would not be required for inhibition by indole. Indene has no effect on the enzyme reaction but the addition of an alanine side chain to give α-amino-β-3-indenylpropionic acid, produces a compound which inhibits tryptophanase action, especially if it is pre-incubated with the enzyme before the addition of substrate. This may reflect a tendency for the side chain of the molecule to combine with the enzyme surface. The results with the two substituted propionic acids, taken together with the previous findings, suggest that combination in the tryptophanase system occurs between the apoenzyme and the carboxyl group of tryptophan. The apoenzyme would consequently be joined to the substrate by a two-point contact, by bonding with the ring nitrogen atom and through the carboxyl group in the side chain.

We are left with the position of the coenzyme pyridoxal 5-phosphate in the complex. The integrity of the α-amino group in tryptophan is essential to the complete tryptophanase system. If this group were attached directly to the apoenzyme we would expect β-3-indolylethylamine, which possesses an unsubstituted ring nitrogen atom and an α-amino group in the side chain, to inhibit the enzyme reaction strongly; in fact its inhibition is very weak. It seems reasonable to assume that β-3-indolylethylamine combines with the coenzyme, and since the tryptophanase reaction is inhibited by carbonyl reagents (Dawes & Happold, 1949) the logical assumption is that combination occurs between the α-amino group of the substrate and the aldehydic group of the pyridoxal phosphate. Combination between pyridoxal phosphate and amino acids during enzymic and non-enzymic reactions has often been suggested, and recently Baddiley (1952) showed that under certain conditions non-enzymic transamination could occur between α-amino acids, pyridoxal phosphate and a metal ion.

Umbreit & Wadell (1949) have shown that deoxypyridoxine, which antagonizes the action of pyridoxal phosphate and pyridoxine with *Streptococcus faecalis R*, has no effect upon the following free enzyme systems: tryptophanase, aspartic-glutamic transaminase and tyrosine decarboxylase. Deoxypyridoxine phosphate, however, inhibited the tyrosine decarboxylase system completely if added to the apoenzyme before the addition of coenzyme. This was taken as evidence that the method of combination between apoenzyme and coenzyme was by means of the phosphate group, possibly by hydrogen bonding, and Beechey & Happold (unpublished results) have now been able to show a similar inhibition when deoxypyridoxine phosphate is added to the apoenzyme of tryptophanase before the addition of pyridoxal 5-phosphate.

Tryptophan and pyridoxal phosphate give rise with tryptophanase to substrate-enzyme and coenzyme-enzyme saturation curves respectively (Wood *et al.* 1947; Dawes & Happold, 1949), and it can be assumed that each forms an intermediate complex with the enzyme resulting probably in a ternary complex. The logical interpretation of the present data suggests that the apoenzyme is bound to the coenzyme through the phosphate group of the latter while the coenzyme is associated with the substrate through the carboxyl group of the pyridoxal phosphate and the amino group of tryptophan, the latter being combined with the apoenzyme through the ring nitrogen atom and the terminal carboxylic acid group.

Finally, Baker (1953) has developed a possible model for the tryptophan-tryptophanase system which is based upon our experimental findings and of our acceptance of α-aminoacrylic acid as an intermediate in the degradation of tryptophan as suggested by Gale (1951). He has constructed molecular models of L- and D-tryptophan and pyridoxal 5-phosphate which show that (*a*) pyridoxal phosphate and tryptophan molecules fit neatly together to form a molecular complex in which the interacting aldehyde group of the former and the amino group of the latter are brought into close juxtaposition, and (*b*) in this complex with L-tryptophan (but *not* with the D-form), the various groups through which we suggest bonding with the apoenzyme occurs, all lie in one plane.

Thus geometry also indicates that, granted the previously mentioned groups are involved in the activity of the substrate-apoenzyme-coenzyme complex, the most probable associations are those we have suggested.

SUMMARY

1. A study of the effect of certain structural analogues of tryptophan on the tryptophan-tryptophanase reaction shows that indole and some indolyl acids inhibited the reaction strongly, while slight inhibition was given by α-amino-β-3-indenylpropionic acid when pre-incubated with the enzyme. Indene, D-tryptophan, β-3-indolylethylamine and β-1-methyl-3-indolylalanine either failed to inhibit or inhibited the reaction but slightly.

2. The enzyme system did not produce indole with any indole-containing substrate other than L-tryptophan, nor was ammonia or pyruvic acid produced from α-amino-β-3-indenylpropionic acid.

3. The relationship of these results to possible mechanisms of the enzyme reaction is discussed. It is suggested that the apoenzyme and coenzyme are linked through the phosphate group of the latter, whilst coenzyme and substrate are joined through the aldehyde group of the coenzyme and the amino group of the substrate. The substrate appears to be combined with the apoenzyme through the ring nitrogen atom and the terminal carboxyl group.

We are grateful to Dr I. C. Gunsalus and Dr W. W. Umbreit for gifts of pyridoxal phosphate, to Prof. Clemo for a sample of α-amino-β-3-indenylpropionic acid and to Dr H. R. Snyder for the homotryptophan. We should also like to thank the Medical Research Council for a grant 'in aid' and a studentship to one of us (H. G.).

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A Note on the Action of Fluoroacetate

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Liébecq & Peters (1949) showed that the inhibitory action of fluoroacetate on the oxidation of pyruvate and fumarate could not be accounted for by the postulated competitive inhibition of acetic acid oxidation (Bartlett & Barron, 1947). They demonstrated that citrate accumulated during the oxidation of fumarate and pyruvate and suggested that fluoroacetate after activation underwent a condensation to form a fluorotricarboxylic acid. This hypothesis has since been confirmed (Buffa, Peters & Wakelin, 1951) and the inhibitor, presumably fluorocitric acid, has been isolated in crystalline form (Peters, Wakelin & Buffa, 1951). These authors state that this is apparently not a simple case of competitive inhibition, because of the straight-line relation which was found between the active substance and the effect upon citrate disappearance. They used however only one concentration of citrate. Although the theory of competitive inhibition can only be applied with reserve to a multienzyme system contained in particles such as mitochondria, it seemed of interest to apply the method of Lineweaver & Burk (1934) to the system; we have therefore examined the effect of fluorocitrate on citrate oxidation by rabbit-kidney mitochondria at various concentrations of substrate and inhibitor. These experiments were done in 1950 during another investigation (Judah & Rees, 1953) and are published here in the hope that the results might be of interest to others.

Methods

Mitochondria from rabbit kidney were obtained by the method of Schneider (1948) using 0·25 M sucrose as the medium.

Oxygen uptake was measured manometrically in a Warburg apparatus in air at 38°. Initial velocities were taken as μl. O_2 uptake in the first 5 min. after 10 min. equilibration.

Citrate was estimated by the method of Weil-Malherbe & Bone (1949).

Materials

Adenosine 5′-phosphate obtained commercially was crystallized twice from hot water. By spectrophotometric analysis (Kalckar, 1947) and paper chromatography (Hanes & Isherwood, 1949) it was pure.

Sodium citrate was a commercial preparation.

Sodium fluoroacetate was provided by Dr R. A. E. Galley of the Agricultural Research Council.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled water.

Composition of the medium: inorganic phosphate 0·025 M, pH 7·4; adenosine 5′-phosphate 0·001 M; $MgSO_4$ 0·0067 M; KCl 0·025 M; cytochrome *c* 1×10^{-5} M; mitochondrial suspension in 0·25 M sucrose; and water to a final vol. of 3 ml.

RESULTS

During investigations on the action of fluoroacetate on citrate oxidation by rabbit-kidney mitochondria we observed that the inhibition was slight in the early part of the experiment and then progressed. Table 1 shows a typical experiment representative

Table 1. *Progressive inhibition of citrate oxidation by fluoroacetate*

System: standard medium as described in text. 10^{-3} M fluoroacetate; citrate, 30 μmoles. Enzyme, rabbit-kidney mitochondria. All present initially.

	O_2 uptake (μl.)	
	Fluoroacetate	No fluoroacetate
1st 10 min.	236	236
2nd 10 min.	95	203
3rd 10 min.	90	210

of five. This result suggested a competitive inhibition, and we decided to apply the methods of Lineweaver & Burk (1934) to our system. A preliminary experiment was carried out to obtain a value for the Michaelis constant K_m.

Fig. 1 shows an experiment in which two concentrations of fluoroacetate and five concentrations of citrate were used. In this experiment citrate was placed in the side arm of the flask while fluoroacetate and 3 μmoles of L-malate were placed in the main compartment to ensure activation of the inhibitor before citrate came into contact with the enzyme system. This arrangement permitted the formation of the active inhibitor, fluorocitrate, so that when citrate was added to the centre compartment of the flask an immediate inhibition of its oxidation was always observed. Fig. 1 shows that the criteria for competitive inhibition are met. These curves were analysed by the method of least squares. This experiment was repeated four times with the same result.

Calculation gives values for K_i between $1{\cdot}1 \times 10^{-4}$ and $2{\cdot}1 \times 10^{-4}$ M. K_m was $4{\cdot}5 \times 10^{-4}$ M. The variations in K_i can probably be explained by the incomplete activation of fluoroacetate and are certainly too high as they relate to the fluoroacetate concentrations rather than to the unknown concentration of the active inhibitor. Sir Rudolph Peters has drawn our attention to the possibility that synthesis of fluorocitrate continues throughout the experiment and that this would make the analysis extremely complex. However, a comparison of a control system such as described in Table 1 with one to which 2:4-dinitrophenol (DNP) was added 10 min. after the beginning of the experiment showed no difference in the degree of inhibition. This suggests that there is no continuous synthesis of fluorocitrate as DNP has been shown (Judah & Rees, 1953) to block the formation of fluorocitrate. Table 2 shows the disappearance of citrate at five concentrations in the presence of 0·002 M fluoroacetate. It will be observed that citrate disappearance varies with the initial citrate concentration. This experiment was done to confirm by chemical means the manometric experiment illustrated in Table 1.

These experiments were carried out at citrate concentrations between 0·01 and 0·0033 M. Buffa *et al.* (1951) were unable to demonstrate a competitive inhibition, but they used a single concentration of citrate. This was 10 μmoles/3 ml., i.e.

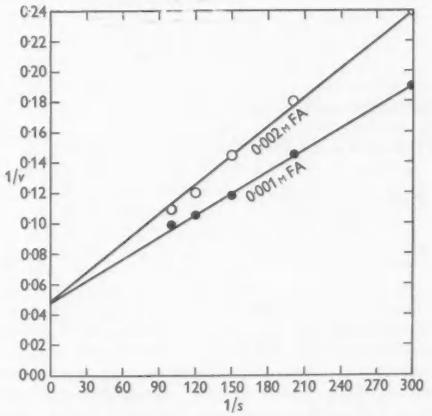

Fig. 1. Demonstration of competitive inhibition between citrate concentrations (S) of 0·01 and 0·0033 M. System: standard medium. Fluoroacetate (FA) was present in the main compartment of the flask, together with 3 μmoles L-malate. Citrate was tipped from the side arm at the end of equilibration (10 min.). $v = O_2$ uptake in first 5 min.

Table 2. *The effect of citrate concentration on citrate disappearance in the presence of fluoroacetate*

System: standard medium as described in text. Fluoroacetate (final concn. 0·002 M) and 3 μmoles of L-malate were placed in the main compartment of the flask. Citrate was tipped from the side arm at the end of equilibration (10 min.). Duration of experiment 20 min.

Citrate concentration (M)	Citrate disappearance (μmoles)
0·01	10
0·0083	7
0·0067	3
0·005	2
0·0033	1

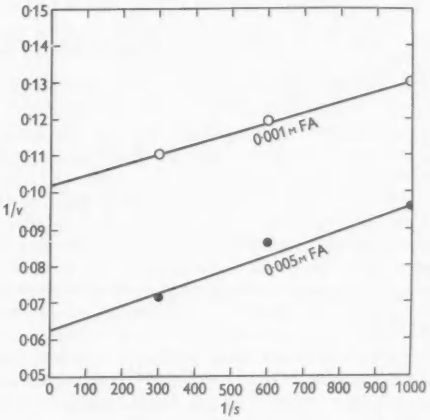

Fig. 2. Demonstration of non-competitive inhibition below citrate concentration of 0·0033 M. Conditions as in Fig. 1. $v = O_2$ uptake in first 5 min. after 10 min. equilibration.

0·0033 M. An experiment carried out with citrate concentrations between 0·001 and 0·0033 M showed non-competitive inhibition. Fig. 2 illustrates this experiment. Though it would appear possible to combine Figs. 1 and 2, this is not permissible as they were obtained from different experiments.

DISCUSSION

Elliot & Kalnitsky (1950) showed a competitive inhibition by fluoroacetate of citrate oxidation by rabbit-kidney preparations. They claim that the oxidation of *cis*-aconitate was not inhibited by fluoroacetate and in fact was accelerated. This result is difficult to explain in view of the findings of Peters & Wilson (1952), who used a soluble aconitase together with a purified sample of the active inhibitor (fluorocitrate) and showed a competitive inhibition of this enzyme by the method of Lineweaver & Burk (1934). They observed that a non-competitive type of inhibition might occur if time were not allowed for the enzyme, substrate and inhibitor to come to equilibrium before measuring the initial velocity of the reaction. We have observed competitive inhibition between citrate concentration of 0·01 and 0·0033 M, whereas below the latter concentration inhibition was non-competitive. We are at present unable to explain this result.

SUMMARY

1. The effect of fluoroacetate on citrate oxidation by mitochondrial systems has been investigated.

2. The apparent competitive nature of this inhibition has been demonstrated with citrate concentrations of 0·01–0·0033 M.

3. Below 0·0033 M citrate concentration the inhibition is non-competitive.

The authors wish to express their gratitude to Prof. E. Baldwin and Prof. G. R. Cameron, F.R.S., for their interest in this work and their constant encouragement.

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Studies in Carotenogenesis

11. CAROTENOID SYNTHESIS IN THE ALGA *HAEMATOCOCCUS PLUVIALIS*

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Haematococcus pluvialis is a unicellular, motile, green alga of the class Chlorophyceae. Like that of most flagellates its resting stage is characterized by the production of cysts, the formation of which is generally regarded as a response to unfavourable environmental conditions, although this may not always be so (see Fritsch (1935) for a full discussion). Formation of cysts in algae is very often accompanied by a change in colour from green to orange or red, and it was in the naturally occurring (red) encysted form of *H. pluvialis* that astaxanthin (3:3′-diketo-4:4′-dihydroxy-β-carotene) was first found in plant tissues (Tischer, 1937, 1944); it was accompanied by small amounts of α- and β-carotenes and lutein. Until that time astaxanthin had been found only in animal tissues, but since then it has also been found in the, presumably encysted, algae, *Brachiomonas simplex* and *Protosiphon botryoides* (Bielig, 1947). (It should be noted, however, that Strain (1951) believes that there are two ketonic carotenoids in *P. botryoides*, neither of which is astaxanthin.)

Another well-known example of a red-encysted alga is *Trentepholia aurea*, the resting stage of

which is very rich in β-carotene (Tischer, 1936; Lederer, 1938; Heilbron, 1942). This pigment was first observed in *T. aurea* by Cohn (1861) who called it haematochrome. This name has also generally been applied to the orange-red pigment from *H. pluvialis* and to the red pigment in the eye spot (stigma) of many flagellates. As we now know that the pigment of *T. aurea* is different from that of *H. pluvialis* and, as there is no direct evidence that the eye-spot pigment is a carotenoid, it is probable that the term haematochrome has served its purpose and should be discarded.

No quantitative observations have been made on the synthesis of astaxanthin by *H. pluvialis*, but it is generally assumed that nitrogen deficiency is one of the major controlling factors (Pringsheim, 1914; Chodat & Mayer, 1927; Meier, 1929; Dr M. Droop, personal communication). On the other hand, Lwoff & Lwoff (1930) found that addition of acetate to a mineral-peptone medium stimulated the synthesis of the red pigment (astaxanthin), although the culture was growing rapidly. Lwoff & Lwoff (1930) also found that no astaxanthin was synthesized in the dark except in the eye spot but, as indicated above, it is not legitimate, at the moment, to conclude that the pigment of the eye spot is a carotenoid.

The work to be described here was undertaken to find out the nature of the carotenoids in the green form of *H. pluvialis*, and to follow quantitatively the synthesis of astaxanthin in the light of present knowledge concerning carotenogenesis in other organisms (Goodwin, 1953).

EXPERIMENTAL

Cultures. The culture of *H. pluvialis* used was obtained from the Type-Culture Collection of Algae and Protozoa, Botany School, Cambridge. Two culture media were used. *A* was 'soil-extract' medium made by extracting soil (1 kg.) with boiling water (1500 ml.) for 15 min. *B* was the medium described by Ondratschek (1940) as giving good growth; it contains (all %, w/v): K_2HPO_4, 0·001; $MgSO_4.7H_2O$, 0·001; $CaCl_2$, 0·001; $FeSO_4.7H_2O$, 0·0001; KNO_3, 0·1 and sodium acetate ($C_2H_3O_2Na.3H_2O$), 0·2; the pH was adjusted to 7·6 with 0·1 N-NaOH.

Cultures were grown in 25 ml. of medium in 100 ml. Erlenmeyer flasks held at 25°, either in a conventional incubator (in the dark), or in a thermostatically controlled water bath illuminated at a distance of 20 cm. by two 80 w 'daylight' fluorescent tubes. Inoculation of the flasks was carried out with 1 ml. of a 3-day 'green' culture grown on the soil-extract medium. It was interesting to find that such inocula would grow when transferred either to a soil extract or KNO_3:acetate medium, but that inocula from a KNO_3:acetate culture would not grow easily when transferred to a soil-extract medium, although it would do so when transferred to a KNO_3:acetate medium.

Extraction, separation and determination of pigments. After a number of trials the following method was found best for extracting carotenoids from *H. pluvialis* and other algae. The cultures were harvested by centrifuging and the residual medium discarded. Aqueous KOH (1 ml. of 60%, w/v) and then ethanol (10 ml.) were added, the centrifuge tube was placed in a water bath at 40° and the mixture constantly stirred and ground with a flattened glass rod. After 5 min. the residue was allowed to settle and the supernatant decanted. The procedure was repeated twice with ethanol (10 ml.) only, when all the pigment was extracted leaving only a very pale yellow residue. The ethanol extracts were combined in a separating funnel and an equal volume of ethyl ether added, followed by water, slowly, until two layers were formed. All the carotenoids except astaxanthin, which had been oxidized to astacin by the treatment just described, were extracted into the ether layer. The potassium salt of astacin accumulated at the ether–aqueous ethanol interface as a fluffy brownish red deposit and the partly degraded chlorophyll was in the aqueous phase.

The ether layer was collected, washed free from alkali with successive small quantities of warm water, dried by the addition of anhydrous Na_2SO_4, separated from this by filtration, and then taken to dryness *in vacuo* at 30°. The residue was then dissolved in a small volume of light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) ready for chromatographic analysis.

The insoluble potassium salt of astacin was collected, dissolved in a small volume of glacial acetic acid and extracted into ethyl ether. After being washed free from traces of acetic acid, the ethereal solution of astacin was treated in the same way as described for the neutral carotenoids.

The neutral carotenoids were chromatographed on either activated alumina (grade 'O'; P. Spence and Co., Widnes) or deactivated alumina, made by treating grade 'O' material with methanol (Goodwin, 1952*a*). Quantitative determinations of the separated pigments were carried out by dissolving the pigment fractions in known volumes of light petroleum and measuring the $E_{1\,cm.}$ value at the appropriate $\lambda_{max.}$. By comparing these values with the known $E^{1\%}_{1\,cm.}$ values for the pure pigments (Goodwin, 1952*b*), the amounts present could easily be determined.

RESULTS

The carotenoids present

In the green stage. The carotenoid extract from 5-day 'soil-extract' cultures were first examined by chromatography on deactivated alumina, and the resulting separation is described in Table 1. It will be seen that only two main zones exist, *A* and *D*. From its adsorptive power and absorption spectrum *D* appeared to be almost certainly lutein. This was confirmed by comparison in a mixed chromatogram with an authentic specimen of lutein; no separation of the pigments could be obtained. Pigments *B* and *C* were present in traces (less than 1% of the total) and could not be identified. Pigment *B*, however, did show some resemblance to echinenone. Fraction *E* (about 5% of the total) is, from its absorption spectrum and high adsorptive power, possibly violaxanthin. There are, however, a number of very strongly adsorbing xanthophylls with very similar absorption spectra (see Strain, 1951). As

Table 1. *Chromatography of carotenoids from green* H. pluvialis

Adsorbent, weakened alumina. Zones in order of increasing adsorptive power. Developer, light petroleum containing various concentrations of ethyl ether. Alga (5 days) cultured at 25° in soil extract.

Zone	Colour	Approx. amount (% of total)	Values of $\lambda_{max.}$ in light petroleum (mμ.)	Concn. of ether (v/v) required to elute zone	Identification
A	Brownish	10–20	~425, 488, 475	0	Mainly β-carotene
B	Pink	<1	453	10	?
C	Yellow	<1	—	15	—
D	Lemon-yellow	75–80	424, 443, 471	80	Lutein
E	Yellow	5	415, 438, 467	100	Violaxanthin?

~ denotes an inflexion.

these pigments are only separable with difficulty and after considerable 'trial and error' chromatography, much more material would have been required for unequivocal identification than was at our disposal.

Fraction *A*, which ran straight through a column of deactivated alumina, was rechromatographed on activated alumina. It consisted essentially of one pigment, β-carotene, identified in the usual manner. Small amounts of *neo*-β-carotene *B* were also observed, but this pigment was probably an artifact, for it has been noticed repeatedly that even when great precautions are taken to eliminate *trans* → *cis* isomerization, it is almost impossible not to produce traces of *neo*-β-carotene *B* when manipulating a solution of 'pure' β-carotene. No α-carotene was present and, furthermore, no colourless polyenes such as phytofluene or phytoene could be detected in the amounts of material examined.

In the red stage. The neutral carotenoids extracted from red *H. pluvialis* were identical with those found in the green cultures but astaxanthin was also present. It was converted into astacin by alkali, and this pigment had an absorption spectrum identical with that of authentic astacin (Goodwin & Srisukh, 1949). As stated in the introduction, however, the presence of this pigment in red *H. pluvialis* has previously been unequivocally identified by isolation in the crystalline state from large naturally occurring encysted cultures.

In the brown stage. The half-way stage between the green and red cultures was examined because of the possibility that it might yield intermediates in the synthetic pathway to astaxanthin; no such pigments had been observed in the red cultures. The same results were, however, obtained in the brown as in the red cultures, except that less astaxanthin was present. No intermediates were noted. It is, of course, more than probable that brown cultures are so coloured because not all the cells have encysted and that the colour is due to a mixture of green and red cells, rather than that they represent an intermediate stage in the physiological process of encystment.

Quantitative experiments

The synthesis of the various pigments during the development of cultures grown on soil extract is illustrated by Table 2. It will be seen that the transition from green to red is accompanied by a considerable synthesis of astaxanthin but that this pigment is not produced at the expense of the neutral pigments which, in fact, also increase in older cultures. In red cultures, the astaxanthin represents about 50 % of the total pigment present. It was found that if green cultures were centrifuged, washed with sterile water, and resuspended in water only, they were still capable of changing into their red form.

Using the KNO_3:acetate medium, the results recorded in Table 3 show that the synthesis of astaxanthin is less in this medium than on the soil-extract medium, although the synthesis of the neutral pigments is increased; astaxanthin accounts for only between 10 and 15 % of the total pigments present. Onset of synthesis of astaxanthin on the KNO_3:acetate medium was delayed until the 11th compared with the 5th day on the soil-extract medium, and was not complete until 14 days after inoculation. Although no dry-weight determinations were carried out, the increased pigment production on the KNO_3:acetate medium appeared, from visual inspection of the cultures, only to be a reflexion of stimulated growth, and not due to a specific effect on pigmentation. As in the case of soil-extract cultures, green cultures washed and resuspended in water only, synthesized astaxanthin.

Effect of light. When 5 to 6-day soil-extract cultures or 10 to 11-day KNO_3:acetate cultures were placed in the dark the colour did not change from green to brown or red. Quantitative experiments on a number of such KNO_3:acetate cultures showed that they had actually lost on the average 67 % of their neutral carotenoids 9 days after being placed in the dark.

Effect of variation in acetate concentration. In an experiment carried out to test the effect on pigment synthesis of the concentration of acetate in the

Table 2. *Carotenoid synthesis by developing* H. pluvialis

Pigment produced per flask containing 25 ml. of soil extract. Cultures from four flasks were combined for each analysis. Incubation at 25°.

Age of culture (days)	Colour	Total neutral carotenoids (μg.)	β-Carotene (μg.)	Astaxanthin (μg.)	Astaxanthin as % of total pigments
5	Green	3·5	0·4	0	0
8	Brownish red	4·4	0·4	6·3	58·9
11	Red	7·0	0·4	9·1	56·5
14	Red	8·2	0·7	9·3	53·1
17	Red	11·3	0·6	8·7	43·5
20	Red	10·5	1·0	9·3	47·0
23	Red	10·7	1·0	10·4	49·3

Table 3. *Carotenoid synthesis by developing* H. pluvialis

Pigment produced per flask containing 25 ml. of KNO_3: acetate medium. Cultures from four flasks combined for each analysis. Incubation at 25°.

Age of culture (days)	Colour	Total neutral carotenoids (μg.)	β-Carotene (μg.)	Astaxanthin (μg.)	Astaxanthin as % of total pigments
5	Green	3·6	0·7	0	0
8	Green	13·8	2·4	0	0
11	Brown	17·8	2·8	1·6	8·3
14	Brownish red	25·8	3·2	4·6	15·1
17	Brownish red	25·6	5·0	2·9	10·3
20	Brownish red	22·3	4·5	3·2	12·6
33	Brownish red	22·7	3·7	3·4	13·0

Table 4. *The effect of acetate concentration on carotenoid production by* H. pluvialis

KNO_3: acetate medium with the acetate concentration varied; concentrations above 0·4% up to 3·0% (w/v) did not support growth. Pigment produced per flask containing 25 ml. of medium. Cultures from four flasks combined for each analysis. Incubation at 25°.

	Green, 8-day cultures			Brown-red, 15-day cultures		
		Astaxanthin			Astaxanthin	
Concn. of acetate (%, w/v)	Total neutral carotenoids (μg.)	μg.	% of total pigments	Total neutral carotenoids (μg.)	μg.	% of total pigments
0	Too small to measure			4·6	<1	—
0·2	8·4	0	0	23·0	4·5	16·3
0·4	2·7	0	0	2·7	<1	10·0

KNO_3: acetate medium it was found (Table 4) that 0·2% (w/v) gave best growth and that above 0·4% no growth was obtained and the cultures appeared to be dead; at 0° little if any growth occurred. Concentrations of acetate between 0 and 0·2% were not examined.

Effect of diphenylamine. Diphenylamine has been shown to be a potent and specific inhibitor of carotenogenesis in *Mycobacterium phlei*, *Phycomyces blakesleeanus* and *Rhodospirillum rubrum* (Turian, 1951; Goodwin, 1952*a*; Goodwin, Jamikorn & Willmer, 1953; Turian & Haxo, 1952); it was, therefore, tested on *H. pluvialis*. This organism was extremely sensitive to diphenylamine and it was not possible to obtain growth even when the diphenylamine concentration was reduced to 1/560 000, a concentration 8–16 times less than normally found effective in the other organisms.

Other possible inhibitors were not tested in great detail because of difficulties in producing cultures on a large scale, but the following (in the concentrations stated) were lethal to washed 7 to 9-day-old suspensions of *H. pluvialis*: arsenite, 3×10^{-4} M; arsenate, 10^{-4} M; NaF, 10^{-4} M; KCN, 10^{-4} M; NaN_3, 10^{-3} M; hydroxylamine, 10^{-5} M; hydrazine, 10^{-3} M; malonate, 4×10^{-4} M; 2, 4-dinitrophenol and sodium iodoacetate, 10^{-4} M.

DISCUSSION

The pigments identified in the green stage of *H. pluvialis* show that in this respect the organism is typical of the Chlorophyceae (Goodwin, 1952*a*), producing only β-carotene and a very simple mixture of xanthophylls (mainly lutein and one other, possibly violaxanthin). These observations

give further confirmation of the generalization that the xanthophylls of the Chlorophyceae are very much less complex than those in the green leaves of higher plants.

No colourless polyenes such as phytofluene and phytoene were observed in this investigation, although their presence in minute traces must remain a possibility if it is assumed that the Chlorophyceae are closely related, from the pigment point of view, to green tissues of higher plants. Recently, colourless polyenes have been observed in traces in leaves using extremely large amounts of starting material (Rabourn & Quackenbush, 1953; Eny, 1953; Zechmeister & Karmakar, 1953). The demonstration of the presence of β-carotene and lutein, as well as astaxanthin in the encysted form of *H. pluvialis* confirms the earlier observations of Tischer (1944). Tischer, however, reported that β-carotene and lutein were present in traces only. This may be so in the naturally occurring encysted forms examined by Tischer, but in our laboratory cultures growing on a nutritionally poor medium (soil extract) the amount of astaxanthin was never more than 60% of the total carotenoids, although the cultures were bright red. The disposition of the astaxanthin in the cell must be such that it masks the yellow of the neutral carotenoids and, incidentally, chlorophyll which we also found in the encysted forms.

The demonstration that the synthesis of astaxanthin does not occur at the expense of the neutral carotenoids indicates that it must be formed from either a colourless precursor already present in the green cultures or from carbon dioxide photosynthetically. At the moment there is no means of deciding which is correct, but the observation that the synthesis of astaxanthin does not take place in the dark indicates that if a precursor is already present, insufficient energy is available from the respiration of the cells to convert it into the pigment, and that this energy must be provided photochemically. In this respect it is interesting to compare the synthesis of astaxanthin in *H. pluvialis* with that of spirilloxanthin in *Rsp. rubrum* originally grown in the presence of diphenylamine (Goodwin & Osman, 1954). These diphenylamine cells, which are almost colourless, will, when washed free from diphenylamine and resuspended in phosphate buffer, synthesize spirilloxanthin provided energy is available. This can be provided photochemically in anaerobic cultures or by respiration in dark-aerobic cultures.

As stated previously, it is now generally assumed that encystment and astaxanthin production is a result of a lack of assimilable nitrogen. The present results, which show that in the nitrogen-poor medium (soil extract) *H. pluvialis* produces relatively more astaxanthin than in the nitrate:acetate medium *B*, tend to confirm this view. There are, however, other factors, such as the nature of the sources of nitrogen, which may eventually also be shown to be important.

It would be unwise to conclude from the results shown in Table 4 that the addition of acetate to the basic nitrate medium (medium *B* without acetate) specifically stimulated synthesis of neutral carotenes and astaxanthin, because growth, as judged by the appearance of the culture and the size of the centrifuged cell mass, was also stimulated considerably; in fact, very little growth occurred in the absence of acetate. It would also be unwise to compare the present results, obtained using nitrate as the source of nitrogen, with the early qualitative observations of Lwoff & Lwoff (1930) that addition of acetate stimulated astaxanthin synthesis when peptone was the source of nitrogen. Our *H. pluvialis* would not grow on medium *B* in which potassium nitrate was replaced by peptone.

SUMMARY

1. Green cultures of *Haematococcus pluvialis* contain β-carotene, lutein and small amounts of a third pigment, possibly violaxanthin. Traces of two other pigments were observed but no colourless polyenes could be detected. Lutein represents about 75–80% and β-carotene 10–20% of the total carotenoids.

2. The change in cells of *H. pluvialis* from green to brown or red is accompanied by the formation of considerable amounts of astaxanthin. This synthesis is not at the expense of the pigments already present in the green stage.

3. Relatively more astaxanthin is formed on a soil extract medium than on a nitrate:acetate medium.

4. Light is necessary for the formation of astaxanthin.

5. Diphenylamine completely inhibits growth of *H. pluvialis* at a concentration of 1/560000.

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The Effect of 3:5-Dinitro-*ortho*-cresol on Phosphocreatine and the Adenosine Phosphate Compounds of Rat Tissues

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A distinctive feature of poisoning by dinitro-*o*-cresol (DNOC) in man (Luijt, 1947) and in experimental animals (Parker, Barnes & Denz, 1951) is the extreme muscular rigidity which develops at or very soon after death. Stoner, Threlfall & Green (1952) have shown that this muscular change in DNOC poisoning is accompanied by a decrease in the concentration of polyphosphates, phosphocreatine and glycogen, and drew attention to the similarity of these changes to those described by Bate-Smith & Bendall (1949) and Bendall (1951). In the present study preliminary experiments indicated that in certain circumstances, death of DNOC-poisoned rats could occur when relatively normal amounts of energy-rich phosphate compounds were present in the muscle. At the same time these animals exhibited the typical rapid onset of rigor. In consequence, this point was investigated more fully and, as changes in phosphate metabolism might well have been expected in other tissues, the observations were extended to the heart, brain and diaphragm.

METHODS

Procedure

In the experiments involving the analysis of muscle it was necessary to minimize the death struggles of the animals, as such movements increase the rate of breakdown of high-energy phosphate compounds. This purpose was achieved by the use of the muscle-relaxing drug, α:β-dihydroxy-γ-(2 methylphenoxyl)propane (Myanesin, British Drug Houses Ltd.), following the suggestion of Bate-Smith & Bendall (1948). The animals (unless otherwise stated the animals used were albino female rats of 200 g. wt.), were given an intraperitoneal injection of Myanesin (200 mg./kg. body wt.) 10 min. before administration of DNOC. At the time of injection of DNOC the animals were completely relaxed. Normal values for phosphate compounds were obtained from untreated animals. These rats were given an injection of Myanesin sufficient to produce relaxation in 10 min. (200 mg./kg. body wt.) and then killed 5–15 min. after this time by a blow on the head, or, if the brain was required, by immersion in liquid O_2. A group of eleven rats was used for muscle analysis (group IV in Tables 1 and 3) and six of these were used for brain analysis. The values of phosphate compounds of heart and diaphragm were obtained from a second group (group VII in Tables 2 and 3) of rats treated similarly to the first group.

DNOC was administered by intraperitoneal injection of a 1% (w/v) solution of the sodium salt adjusted to pH 7·4.

Immediately after death, the treated animals were held head downwards in liquid O_2. The head and shoulders were immersed, but freezing of the rest of the body was avoided in order to ease the removal of the heart and diaphragm. After the initial strong ebullition had subsided, the animal was removed from the liquid O_2 and decapitated by a single blow from a broad-bladed chisel. The head was immediately returned to the liquid O_2. It was found that if the head was left in liquid O_2 for at least 3 or 4 min. the skull usually cracked in an anterior-posterior direction. This enabled the skull to be split into two roughly equal portions each containing half of the brain. Immediately after decapitation, samples of muscle weighing between 1 and 2 g. were cut from one of the hind limbs and dropped into liquid O_2. The heart was removed whole and washed in ice-cold water before being placed in the liquid O_2. The diaphragm was removed as completely as possible, washed quickly in ice-cold water and then frozen.

Preparation of extracts

Skeletal muscle. The frozen muscle was weighed and ground with washed sand in 3×5 ml. of 3% (w/v) perchloric acid. The filtered extracts were combined and neutralized with N-NaOH. The solution was refiltered and made up to 50 ml. with distilled water.

Heart muscle. The heart was weighed and ground with sand in 2 × 5 ml. of 3% perchloric acid. The combined extracts were centrifuged on a bench angle-head centrifuge for about 30 sec. The supernatant was decanted, neutralized with N-NaOH, made up to 20 ml. and filtered.

Diaphragm. The weighed diaphragm was powdered in a stainless-steel mortar previously cooled in liquid O_2. The powder was tipped into 5 ml. of ice-cold 3% perchloric acid and stirred with a glass rod for about 1 min. The extract was filtered and the residue re-extracted with 5 ml. of 3% perchloric acid. The second extract was filtered and combined with the first. The combined extracts were neutralized with N-NaOH, filtered and made up to 20 ml. with distilled water.

Brain. As much as possible of the whole brain was chipped from the head and, after weighing, was ground with washed sand in 2 × 5 ml. of 3% perchloric acid. The combined extracts were centrifuged for approximately 30 sec. The supernatant was decanted, neutralized with N-NaOH, made up to 20 ml. with distilled water and filtered. Portions of the extracts were taken for analysis.

Inorganic phosphate (I.P) was determined by the method of Berenblum & Chain (1938) as modified by Ennor & Stocken (1950). Phosphocreatine phosphate (CP.P) was regarded as the increment of inorganic phosphate obtained by leaving a portion of the extract in contact with the acid molybdate reagent for 30 min. Total energy-rich phosphate (~P) was determined as the phosphate liberated by hydrolysis in N-HCl at 100° for 7 min. and was taken to include the labile phosphate from adenosine triphosphate, adenosine diphosphate and phosphocreatine (ATP + ADP + CP.P). In a few extracts total phosphate was determined essentially by the method of LePage (1951). Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were determined by the enzymic method of Kalckar (1947).

The general experimental procedure described above was modified in one case where it was required to estimate the loss of energy-rich phosphate compounds after death. Male rats of 350 g. wt. were used. Myanesin and DNOC were administered as before. Immediately after death the animals were severed below the ribs to prevent the possibility of post-mortem circulation, and 4–5 g. of muscle were removed from the right hind limb. The sample of muscle was placed in liquid O_2 to wait analysis and the lower portion of the body bearing the left leg placed in an incubator at 37° for 15 min. A sample of muscle was then cut from the left leg and frozen in liquid O_2. The muscle samples were treated separately as follows. The muscle was weighed, ground with washed sand with 3 × 5 ml. of 3% perchloric acid. The filtered extracts were combined and made up to 25 ml. with distilled water. 5 ml. of this solution were neutralized with N-NaOH and used for the estimation of total energy-rich phosphate. The remaining 20 ml. were used for the determination of acid-soluble glycogen as follows. An equal volume of cold ethanol was added to the extract. The following morning, the precipitated glycogen was centrifuged, the supernatant drained off and the residue hydrolysed with N-H_2SO_4 in a boiling-water bath. Glucose was determined by the method of Hagedorn & Jensen (1923) and the result expressed as glucose equivalent of glycogen.

Denervation experiment. Three rats were used. Each rat was placed under ether anaesthesia and the sciatic nerve cut at the point where it entered the left thigh. The nerve supply to the right leg remained intact. Five hours afterwards the rats were given an intraperitoneal injection of DNOC. The time of death and of onset of rigor were noted. Dr J. M. Barnes performed the operation on the rats for this experiment.

RESULTS

Concentration of energy-rich phosphate compounds after a lethal dose of DNOC

Tables 1 and 2 summarize the effect of high doses of DNOC upon the phosphate compounds in muscle, brain, heart and diaphragm. The size of the dose appeared to have no effect other than on the time of survival so the experiments have been grouped according to survival times. Death in most of the rats was preceded by spasmodic stretching of the limbs together with a few slow gasps which ceased when the heart beats could no longer be felt. The exact moment when death occurred was difficult to determine: with the exception of one group, intense stiffening of the body was obvious within a few seconds of the final heart beat. In some cases, a stiffening of the hind limbs could be felt just before death. The exceptional group was that which heads Table 1. In this experiment the rats died very shortly after the injection of DNOC and were limp at death. However, with other rats which were given a similarly high dose of DNOC, rigor set in a few minutes after death. Untreated rats killed by a blow on the head were not in rigor until the lapse of 3 or 4 hr. and the rigor did not appear to be as marked as that of DNOC-poisoned rats.

From the tables it is seen that in the rats of group I there is a diminution of CP.P in muscle and at the same time almost total loss of this compound from the brain. The amount of ATP in muscle is not significantly lower than that of the control animals ($t = 0{\cdot}7$, $P = 0{\cdot}49$), but again there is very little of this compound in the brain. Because of an increase of AMP the concentration of total adenine compounds has remained as high as in the untreated rats. With a slightly longer survival time, group II, there is an increased loss of muscle CP.P but no further change in the ~P compounds of the brain. CP.P has virtually disappeared from the muscles of group III (those which survived for an hour or more) and there is a decrease of approximately 50% in the amount of ATP as well as a loss of ADP. The level of phosphate compounds in the brains of this group remains essentially the same as before. In all these groups a loss of ~P is paralleled by a gain in I.P. In muscle extracts estimation of total phosphate gave values which were not significantly different from those in extracts of untreated rats ($t = 0{\cdot}7$, $P = 0{\cdot}5$). In general, the level of total ~P in the muscle is inversely proportional to the time taken by the poisoned animals to die. On the other hand, low concentrations of total ~P are reached at the earliest time observed.

Table 1. *Effect of lethal doses of DNOC on energy-rich phosphate compounds of muscle and brain*

Figures in parentheses refer to number of rats used. All values in terms of μmoles/g. wet wt. and given with S.E. ATP, ADP and AMP were determined by the enzymic method. The mean and the standard error of the mean of the total adenine compounds were calculated from the total obtained from each rat. Inorganic phosphate (I.P) and phosphocreatine phosphate (CP.P) were estimated by the inorganic phosphate method.

Group	Dose (mg./kg.)	Survival time (min.)	I.P	CP.P	ATP	ADP	AMP	Total adenine compounds (ATP + ADP + AMP)
				Muscle				
I (3)	80	3–5	12·5±2·6	7·3±1·25	3·93±0·54	2·73±0·39	0·82±0·21	7·48±0·48
II (6)	30–40	8–14	17·6±1·1	4·0±0·52	3·83±0·35	2·44±0·29	0·40±0·20	6·67±0·64
III (9)	17·5–22·5	60–90	25·0±1·3	1·1±0·27	2·45±0·31	0·86±0·14	0·80±0·19	4·11±0·38
IV (11)	Controls	—	6·1±0·8	15·2±0·59	4·54±0·33	2·74±0·31	0·38±0·09	7·66±0·60
				Brain				
I (3)	80	3–5	7·1±1·7	0·64±0·28	0·21±0·12	0·82±0·13	1·35±0·25	2·38±0·15
II (6)	30–40	8–14	8·0±1·4	0·61±0·23	0·34±0·12	0·73±0·18	0·80±0·21	1·87±0·22
III (9)	17·5–22·5	60–90	9·6±1·3	0·47±0·18	0·37±0·07*	0·65±0·20*	1·26±0·21*	2·28±0·24*
IV (6)	Controls	—	4·2±0·66	3·5±0·27	1·80±0·13	0·61±0·10	0·57±0·07	2·98±0·26

* Seven rats.

Table 2. *Effect of lethal doses of DNOC on energy-rich phosphate compounds of heart and diaphragm*

Figures in parentheses refer to number of rats used. All values in terms of μmoles/g. wet wt. and given with S.E. ATP, ADP and AMP were determined by enzymic method. The mean and the standard error of the mean of the total adenine compounds were calculated from the totals obtained from each rat.

Group	Dose (mg./kg.)	Death (min.)	ATP	ADP	AMP	Total adenine compounds (ATP + ADP + AMP)
			Heart			
II (6)	30–40	10–20	0·67±0·08	0·64±0·14	2·63±0·17	3·94±0·25
III (3)	22·5	60	0·58±0·25	0·78±0·25	2·88±0·28	4·24±0·44
VII (6)	Controls	—	1·65±0·03	1·01±0·14	0·65±0·16	3·31±0·33
			Diaphragm			
II (3)	30	10–20	1·59±0·17	0·35±0·07	0·35±0·07	2·29±0·33
VII (3)	Controls	—	2·46±0·25	0·80±0·23	0·38±0·25	3·64±0·25

Table 3. *Concentration of energy-rich phosphate compounds of muscle, heart and brain of rats killed after administration of DNOC*

The rats were killed at the stated times after intraperitoneal injection of DNOC. Figures in parentheses refer to number of rats used. All values in terms of μmoles/g. wet wt. and given with S.E. Analyses of inorganic phosphate (I.P) and phosphocreatine phosphate (CP.P) in heart muscle were not performed. ATP, ADP and AMP were determined by enzymic method. The mean and the standard error of the mean of the total adenine compounds were calculated from the totals obtained from each rat.

Group	Dose (mg./kg.)	Death (min.)	I.P	CP.P	ATP	ADP	AMP	Total adenine compounds (ATP + ADP + AMP)
				Muscle				
V (9)	40–60	5–10	10·6±1·5	7·8±1·6	4·8±0·22	1·20±0·33	0·36±0·02	6·36±0·53
VI (3)	15 (sublethal)	30	9·0±1·7	6·2±0·25	4·8±0·17	0·86±0·33	0·14±0·00	5·80±0·30
IV (11)	Controls	—	6·1±0·08	15·2±0·59	4·54±0·33	2·74±0·31	0·38±0·09	7·66±0·60
				Brain				
V (9)	40–60	5–10	6·1±0·27	0·94±0·27	0·66±0·21	0·78±0·11	0·69±0·26	2·13±0·27
VI (3)	15 (sublethal)	30	7·1±1·3	1·38±0·10	1·12±0·13	0·94±0·10	0·82±0·23	2·88±0·21
IV (6)	Controls	—	4·2±0·66	3·5 ±0·27	1·80±0·12	0·61±0·10	0·57±0·07	2·98±0·26
				Heart				
V (3)	40–60	5–10	—	—	1·6±0·01	0·52±0·10	2·63±0·25	4·75±0·42
VI (3)	15 (sublethal)	30	—	—	1·83±0·17	1·65±0·45	2·20±0·61	5·68±0·50
VII (6)	Controls	—	—	—	1·65±0·03	10·1±0·14	0·65±0·16	3·31±0·33

In Table 2 it is seen that the ~P concentration of the heart falls to a value which is between that of the values in muscle and brain at the corresponding survival times. It will be noticed that the total amount of adenine compounds has increased above that found in the untreated animals. There is some loss of ATP in the diaphragm but not to any greater extent than in skeletal muscle.

In view of the magnitude of the loss of ~P from the brain it was of interest to see if this change could be detected at an early stage after the injection of DNOC. For this purpose rats were treated with lethal or sublethal doses of DNOC and killed after a fixed time interval. Table 3 shows the results obtained. Five to ten minutes after a lethal dose of DNOC a 50 % loss of CP.P was found in the muscle, but with the possible exception of ADP there was no other change. On the other hand, there is a considerable loss of both CP.P and ATP in the brain. In the heart there is no decrease of ATP but there is a rise of AMP and total adenine compounds. The muscle of rats subjected to a non-lethal dose of DNOC and killed 30 min. afterwards shows changes of ~P compounds similar to those obtained with lethal doses. In the brain the concentrations are a little higher than those of the preceding group but are still considerably below the control values. Again the heart shows an increase in AMP concentration.

Catalytic effect of DNOC on rate of loss of ~P

It has been reported that the related compound, 2:4-dinitrophenol, accelerates the hydrolysis of ATP *in vitro* (Witter, Newcomb & Stotz, 1953; Lardy & Wellman, 1953). As this may have been a factor contributing to the breakdown of ATP in DNOC-poisoned rats an attempt was made to compare the rate of destruction in treated and untreated animals. In the normal animal three important metabolic processes counterbalance any loss of ATP. CP.P and ADP form ATP via the Lohmann reaction, some ATP is formed during anaerobic glycolysis, while most is synthesized by aerobic phosphorylation during oxidation of substrates by the Krebs cycle. It was necessary to choose experimental conditions in which these factors were eliminated or allowed for. As described in the experimental section the rats were killed and any possibility of post-mortem circulation minimized. Under these conditions it was assumed that aerobic phosphorylation was virtually absent. Estimation of glycolysis during the experiment made it possible to apply a correction for this factor. As phosphocreatine is convertible into ATP, the total ~P was determined and was regarded as available ATP. From Table 4 it is seen that there is no great difference between the rates of breakdown of ~P in poisoned and unpoisoned rats. It is concluded that acceleration of hydrolysis of ATP by DNOC is not a major factor contributing to the losses observed in the preceding experiments.

Effect of Myanesin

It has been mentioned that the poisoned rats die with a spasmodic stretching of the limbs and that in some cases stiffening of the legs could be detected before death. It was possible that this muscular activity might in some part be due to stimuli from the central nervous system even in the presence of Myanesin. Table 5 shows that Myanesin had no effect upon the onset of rigor but significantly prolonged the survival time. In a second experiment (Table 6) DNOC was administered to rats which had been previously deprived of nervous control of one limb. No Myanesin was used. Some evidence is shown that the presence of intact nervous control accelerated the rate of onset of rigor.

Table 4. *Comparison of loss of energy-rich phosphate in muscle of DNOC-poisoned and untreated rats*

Inorganic phosphate (I.P) and ~P were determined by modified method as described in Experimental section. All values in terms of μmoles/g. wet wt. Muscle of right leg frozen immediately; left leg incubated at 37° for 15 min. prior to removal of muscle.

Rat	Leg	I.P	I.P + ~P	Total ~P	Differences in ~P between legs, i.e. loss of ~P in 15 min.	Glycogen (μmoles glucose)	~P derived from glycolysis in 15 min.	Loss of ~P in 15 min. Corrected for glycolysis
				Controls				
1	R.	5·2	31·0	25·8	5·8	7·0	—	—
	L.	12·5	32·5	20·0		Lost	—	—
2	R.	8·0	33·6	25·6	9·6	12·9	—	—
	L.	17·0	33·0	16·0		11·4	4·5	14·1
3	R.	7·1	27·5	20·4	1·7	10·5	—	—
	L.	9·3	28·0	18·7		3·5	21·0	22·7
				DNOC (50 mg./kg.)				
4	R.	7·7	21·3	13·6	8·4	5·0	—	—
	L.	27·0	32·2	5·2		1·2	11·4	19·8
5	R.	8·4	24·6	16·4	10·2	4·6	—	—
	L.	22·2	28·4	6·2		2·0	7·8	18·0
6	R.	5·5	25·4	19·9	0·2	6·8	—	—
	L.	8·1	25·8	19·7		1·5	15·9	16·1

Table 5. *Effect of Myanesin on DNOC-poisoned rats*

Myanesin given by intraperitoneal route 10 min. before injection of DNOC. Six rats in each group.

DNOC (mg./kg.)	Myanesin (mg./kg.)	Time of death (min.)	Time of rigor after death (min.)
80	None	3·3	0·3
80	200	5·8	0·5

Table 6. *Effect of denervation on time of onset of rigor after DNOC poisoning*

One limb denervated as described in Experimental section. DNOC administered 5 hr. after operation when the animals had fully recovered from ether anaesthesia.

DNOC (mg./kg.)	Time of death (min.)	Time of rigor (min.)	
		Untreated leg	Denervated leg
40	10	0·2	0·2
30	56	0·5	2·3
30	90	0·1	1·8

DISCUSSION

2:4-Dinitrophenol has been extensively used as an agent for 'uncoupling' oxidative phosphorylation *in vitro* (Judah, 1951). The structural similarity of this compound and DNOC and the close similarity in toxic properties of both compounds to men and experimental animals warrants the assumption that DNOC and 2:4-dinitrophenol have a common action. Some evidence for this assumption is available in the work of Clowes, Keltch, Strittmatter & Walters (1950) who used DNOC to inhibit the oxidative phosphorylation of Arbacia eggs, and from a personal communication from Dr J. D. Judah who states that DNOC uncouples the oxidative phosphorylation of liver mitochondria.

It is reasonable to expect that in an animal poisoned by DNOC, inhibition of oxidative phosphorylation would deprive the tissues of their main source of ~P. The post-mortem experiment of Table 4 suggests that acceleration of ATP hydrolysis is not a major factor in the breakdown of this compound *in vivo*. Normal activity in the absence of synthesis therefore exhausts the supply of ~P. In the absence of precise information it is not possible to say to what extent the ~P concentration can fall in a particular tissue before its function is impaired. However, of the tissues examined the brain sustains an early and considerable loss of ~P which may be an important factor in the sequence of events leading to the death of the DNOC-poisoned animal. The accepted role of phosphocreatine is to maintain the level of ATP via the Lohmann reaction. Therefore in the absence of the major synthetic source of energy-rich phosphate bonds, the first indication of a disturbed phosphate metabolism should be the loss of phosphocreatine and such a loss is obvious in the studies of Bendall (1951) and of Stoner *et al.* (1952). In the present experiments, in a short time after the injection of a lethal dose of DNOC, there is a 50% reduction of phosphocreatine concentration in the muscle and a virtual disappearance of this compound as well as of ATP from the brain. After a longer survival time there is a further loss of phosphocreatine and a loss of ATP and ADP from the muscle. In the brain, phosphocreatine and ATP being almost all destroyed in the first 5 min., there is no further change. In this tissue in two groups there is an increase of AMP suggesting that there may be relatively less adenylic acid deaminase in the brain. In the diaphragm and heart there was a loss of ATP of similar magnitude to that found in skeletal muscle of animals which survived approximately the same time but the loss was never as great as that found in the brain. The significance of the increase of AMP and hence of total adenine compounds in the heart is not apparent. The conditions under which the experiments were performed were far from ideal and make interpretation difficult. Pollack, Flock & Bollman (1934) found it necessary to remove the heart under artificial respiration in order to estimate its phosphocreatine content, and similar precautions may be required to obtain an accurate picture of adenosine polyphosphate distribution in the heart. The experiments in which the animals were killed shortly after an injection of DNOC also confirm that the greatest change takes place in the brain.

In introducing these studies it was stated that a remarkable feature of death due to DNOC poisoning was the intense and rapid onset of rigor. The experiments of Stoner *et al.* (1952) and the results for the group of rats in the present series which survived an hour or more appear to confirm the impression that the sequence of events in DNOC poisoning is similar to that in rigor mortis, i.e. a complete loss of phosphocreatine followed by a rapid fall in ATP concentration and the onset of rigor. When the results from the groups of rats which survived only for a short time are examined, it appears, however, that a close analogy between the two types of rigor does not always exist. The muscles of these rats, while suffering a considerable loss of phosphocreatine, contain normal amounts of ATP and ADP and yet are quite rigid. The rigor studied by Bendall (1951) and Lawrie (1953) was measured mechanically and expressed in terms of loss of extensibility. To that extent the present experiments are not comparable because no attempt was made to measure the extent of rigor but only the presence or absence of complete

rigidity as observed by touch was recorded. In most of the animals, death was preceded by a spasmodic stretching of the limbs, and it was thought that a nervous mechanism might play some part in the rigidity, but the experiments on the rats with one denervated limb and on other rats without Myanesin showed that rigor was delayed for only a minute or two. It follows that a fall in ATP concentration is not necessarily a concomitant of the stiffening which immediately follows death by DNOC poisoning. This being so, the physical change observed in skeletal muscle may be paralleled by a similar change in the muscle of the heart and diaphragm. In this connexion it is interesting to note that kymograph recordings of movements of the diaphragm and of the blood pressure of DNOC-poisoned rabbits indicate that respiration and the heart stop almost simultaneously (J. M. Barnes, personal communication).

SUMMARY

1. After lethal doses of 3:5-dinitro-*ortho*-cresol (DNOC), depletion of energy-rich phosphate compounds occurs in skeletal muscle, brain, heart and diaphragm.

2. The extent to which this change takes place in the muscle depends upon the survival time of the poisoned animal, but at all times nearly maximal loss of energy-rich phosphate compounds is found in the brain.

3. The rapid onset of rigor seen after the death of rats poisoned with DNOC is not necessarily associated with subnormal concentrations of adenosine triphosphate in muscle.

I am grateful to Dr J. M. Barnes for performing the denervation experiment and to Miss G. R. Reynolds for technical assistance.

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Formation of Formylkynurenine by the Action of X-Rays on Tryptophan in Aqueous Solution

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It is well known that the action of X-rays on water leads to the production of H atoms and OH radicals according to the net process (Weiss, 1944, 1947):

$$H_2O \rightsquigarrow H + OH, \qquad (1)$$

and, to a much smaller extent, also to a direct decomposition into molecular hydrogen and hydrogen peroxide (Allen, 1952; Weiss, 1953).

Although OH radicals themselves are known to be strong oxidizing agents, recent work has shown that certain specific oxidation processes appear to take place only if molecular oxygen is present in the solution, e.g. in the formation, by irradiation with X-rays, of *o*-quinone from phenol (Stein & Weiss, 1951), nitrite from ammonia (Rigg, Scholes & Weiss, 1952), and hydroxylamine from primary aliphatic amines (Jayson, Scholes & Weiss, unpublished work).

Stein & Weiss (1949*a*) have previously also reported the formation of a dialdehydic substance when aqueous benzene solutions are irradiated with a mixed γ-ray neutron source; the opening of the benzene ring which has been observed under these conditions also takes place with X-radiation when

oxygen is present (Daniels, Scholes & Weiss, unpublished work). The biological conversion of tryptophan into kynurenine, under aerobic conditions (cf. Neuberger, 1944; Dalgliesh, 1951) likewise involves a ring opening, in this case of the indole ring, and this has recently been shown to proceed via the intermediate formation of formylkynurenine (Mehler & Knox, 1950). In the case of *Ephestia* and *Drosophila*, formation of kynurenine from tryptophan was shown to be responsible for the production of eye pigment (cf. Butenandt, 1949).

In view of these observations, it appeared to be of some interest to investigate the effects of X-rays on aqueous solutions of tryptophan, with particular reference to the influence of molecular oxygen. Stenström & Lohmann (1931) have reported that tryptophan is destroyed on irradiation with X-rays. More recently, Proctor & Bhatia (1952) have irradiated tryptophan solutions with high-voltage cathode rays and have measured microbiologically the disappearance of the amino acid. However, no definite chemical changes have been reported by any of these authors.

EXPERIMENTAL

Irradiations. The solutions (100 ml.) contained in Pyrex glass vessels were irradiated with X-rays generated from a Victor Maximar set operating at 200 kv and 15 mA (cf. Farmer, Stein & Weiss, 1949). The dose rate was 2700 r/min. ($1{\cdot}4 \times 10^{17}$ ev/ml. min.) determined by the oxidation of 0·001 M-$FeSO_4$ in 0·8 N-H_2SO_4; for the dosimetry the value of 18 Fe^{3+}/100 ev was used (Weiss, 1953). All solutions were prepared in triply distilled water, ordinary distilled water being redistilled in an all-glass still from alkaline permanganate and then from phosphoric acid.

Irradiations were carried out in the presence of O_2 and also *in vacuo*. Saturation with O_2 was effected by bubbling the gas through the solution for about 20 min. before irradiation. For complete evacuation, the solutions were pumped out by means of a Hyvac oil pump, shaken for a few min. and finally evacuated with a mercury diffusion pump. After this procedure less than 0·01 ml. non-condensable gas remained. Immediately after irradiation the solutions were concentrated by vacuum evaporation at relatively low temperatures (about 25°) or by freeze-drying. The freeze-drying apparatus used was essentially similar to that described by Holzman (1950). In all cases blank solutions were treated in an identical manner.

Paper chromatography. We were essentially concerned with the identification of small quantities of kynurenine and formylkynurenine in the presence of a large excess of tryptophan.

Of the several solvent systems examined, ethanol:sulphuric acid:water (160:1:39 v/v; Hellmann, 1951*a*) proved to be the most effective for the separation of kynurenine under these conditions. The chromatograms were run at 36° using Whatman no. 1 paper and the ascending technique described by Hellmann (1951*b*), at least 30 cm. solvent travel being allowed in all cases; the R_F values of tryptophan and kynurenine were 0·85 and 0·72, respectively. Since in the solvent containing sulphuric acid formylkynurenine was hydrolysed to kynurenine, ethanol:acetic acid:water (155:20:25, v/v) was used for the detection of the formyl derivative, the chromatograms being run at room temperature. Although the separation was not particularly good in this solvent (R_F values for formylkynurenine and tryptophan were 0·20 and 0·23, respectively, formylkynurenine could be readily distinguished by the methods described below.

Paper chromatographic separation of mixtures of both kynurenine and oxindolylalanine (DL-α-amino-β-oxindol-3-ylpropionic acid) from relatively large quantities of tryptophan has been attempted but, as yet, no satisfactory result has been achieved. In the sulphuric acid solvent oxindolylalanine runs to almost the same position as kynurenine and is therefore obscured by it. In the absence of kynurenine, however, oxindolylalanine can be readily detected on the chromatograms.

Two reagents were used for the detection of the substance on the paper, (*a*) the Ehrlich reagent (a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde in 20% (w/v) HCl (Hellmann, 1951*a*)) which gives an orange colour with kynurenine and formylkynurenine, a pale yellow with oxindolylalanine and a violet colour, changing to green, with tryptophan, and (*b*) the Ekman reagent (diazotization followed by coupling with ethyl-α-naphthylamine (Ekman, 1948; Hellmann, 1951*a*)) which gives a carmine red with kynurenine and with formylkynurenine. Known quantities of the substances were chromatographed and the limits of detection determined. As little as 1 μg. of kynurenine or formylkynurenine could be detected by means of the Ekman reagent; the Ehrlich reaction was somewhat less sensitive, about 5 μg. being the lower limit. (More recently it has been pointed out to us by Dr C. E. Dalgliesh that if the Ehrlich reagent is made with less acid (e.g. 5% HCl), kynurenine can be detected in much smaller amounts than 5 μg.) Oxindolylalanine, on the other hand, owing to its weak Ehrlich reaction, could not be readily detected in quantities of less than about 15 μg. In the actual irradiation experiments these limits corresponded to about 0·2 μmole formylkynurenine (or kynurenine) and about 15 μmoles oxindolylalanine/100 ml.

The identity of the substances on the chromatograms were, in some cases, confirmed by their absorption spectra. For this purpose the spots were eluted with 5 ml. phosphate buffer (0·02 M Sørensen buffer, pH 7·2) and the spectra recorded between 230 and 400 mμ. by means of a Unicam Spectrophotometer. The spots were located by markers and also by their fluorescence in ultraviolet light in which formylkynurenine and kynurenine exhibit a blue fluorescence.

Hydrolysis of the eluates was carried out in N-HCl at 100° for 10 min. (Mehler & Knox, 1950). The cooled solutions were neutralized with NaOH and again buffered before spectroscopic analysis.

RESULTS

Irradiation of tryptophan

(1) *In the presence of oxygen.* Unbuffered 0·1% (w/v) DL-tryptophan (Roche Products Ltd.) solutions (100 ml.) were irradiated with a total dose of $1{\cdot}6 \times 10^5$ r. After irradiation the solutions exhibited a yellow colour which intensified on standing (cf.

Proctor & Bhatia, 1952). In the preliminary experiments the solutions were concentrated to a small volume by evaporation under reduced pressure. The resulting dark brown concentrate was then subjected to chromatographic analysis, using the ethanol:water:sulphuric acid solvent. Examination of the dried chromatogram under ultraviolet light showed that a considerable number of fluorescent substances had been formed on irradiation. The R_F value of one of the fluorescent spots corresponded to that of kynurenine and gave the colour reactions of this substance with both the Ekman and Ehrlich reagents. The absorption spectrum of the eluted spot was identical with that of kynurenine, except that the second maximum was at 355 mμ. instead of at 360 mμ. However, it was found, confirming an observation of Dalgliesh (1952), that an authentic specimen of kynurenine run on the chromatogram also showed the same depression of this maximum.

The presence of hydrogen peroxide in the irradiated tryptophan solutions was shown by means of the titanium sulphate reagent (Eisenberg, 1943). In view of the possibility that the observed kynurenine may have arisen from some oxidation by the hydrogen peroxide during the concentration of the solution, the irradiated solutions were concentrated by freeze-drying, a process in which the hydrogen peroxide could be completely removed from the system at low temperature. However, chromatographic analysis of the freeze-dried material still showed the presence of kynurenine, thus confirming that this substance was not due to any such secondary attack. In addition, blank experiments were carried out in which tryptophan and hydrogen peroxide (in concentrations equivalent to those present during irradiation) were evaporated together: no formylkynurenine or oxindolylalanine could be detected in the residues. Concentration by freeze-drying was used in all but the preliminary experiments.

Since it was found that formylkynurenine was hydrolysed to kynurenine on chromatograms run in the solvent which contained sulphuric acid it had to be ascertained which of these compounds was, in fact, the initial product of the irradiation. The freeze-dried irradiated tryptophan mixture was therefore chromatographed in an acetic acid solvent, in which it had been shown that no decomposition of formylkynurenine took place. The fluorescent area which gave the Ekmann reaction was eluted and absorption spectra taken before and after acid hydrolysis. From the fact that the absorption band at 355 mμ. appeared only after acid hydrolysis, we have concluded that formylkynurenine is a product of the irradiation of tryptophan solutions in the presence of oxygen.

The yield of formylkynurenine was determined by measuring the amount of kynurenine formed by acid hydrolysis, i.e. the increase in absorption at 355 mμ. A known volume of the concentrated irradiated mixture was run in the acetic acid solvent, and optical densities of the eluates taken before and after hydrolysis in N-HCl for 10 min. The amount of formylkynurenine formed was 2·5 μmoles/100 ml., corresponding to a yield G (molecules of formylkynurenine formed per 100 ev), of the order, $G \simeq 0{\cdot}2$.

Owing to the experimental difficulties described above, it was impossible to determine with certainty whether or not oxindolylalanine was formed in the irradiation. It was, however, established by separate experiments that, had any oxindolylalanine been formed, it would not have decomposed during the working-up procedure.

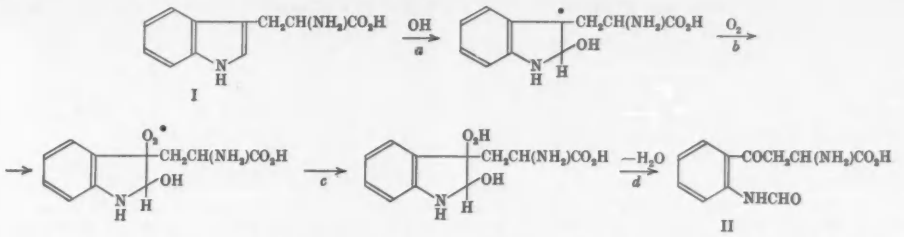
(2) *In vacuo*. Evacuated 0·1 % (w/v) unbuffered tryptophan solutions were irradiated with a total dose of $1{\cdot}3 \times 10^5$ r; only an extremely faint yellow coloration was produced. The solutions were freeze-dried and chromatographed in both the sulphuric and acetic acid solvents. No kynurenine or formylkynurenine could be detected by colour reactions or by ultraviolet spectroscopy; the Ehrlich reaction for oxindolylalanine was also negative.

Irradiation of oxindolylalanine

Unbuffered oxindolylalanine solutions (0·03 %, w/v) were saturated with oxygen and irradiated with a dose of $1{\cdot}3 \times 10^5$ r. As in the tryptophan experiments a yellow coloration was produced. The solutions were freeze-dried and chromatographed in both solvent mixtures. The dried chromatograms when examined under ultraviolet light, showed a somewhat different fluorescence pattern from those of the irradiated tryptophan chromatograms; a very faint Ekman reaction was observed, but the amounts giving this reaction were too small for any further identification procedure.

DISCUSSION

The chemical action of ionizing radiations in dilute aqueous solutions can be attributed to the free OH radicals and H atoms formed by the splitting of the water molecules according to equation (1). The G value for the production of radicals is about 4 (Weiss, 1953). It has been found that G for the formation of formylkynurenine, by irradiation of tryptophan in the presence of oxygen, is only of the order of about 0·2. The radicals, therefore, evidently attack more than one site in the tryptophan molecule: in fact, the relatively complicated nature of the paper chromatograms indicates that a number of different reactions take place during the irradiation. One such process results in the formation of



ammonia (under the above experimental conditions with a yield of $G_{NH_3} \simeq 0{\cdot}45$) and this must involve attack on the alanine side chain, it being well known that amino acids, in general, liberate ammonia on irradiation of their aqueous solutions (cf. Stein & Weiss, 1949*b*; Dale, Davies & Gilbert, 1949). Further, it is very likely that the free radicals will also attack the aromatic ring, leading to the formation of phenolic compounds (cf. Stein & Weiss, 1949*c*); in fact, there were definite indications that phenolic compounds were formed.

Regarding the mechanism of the formation of formylkynurenine it seemed of interest, in the first place, to ascertain whether this involved intermediate formation of oxindolylalanine. This possibility could not be ruled out, since it could not be established with certainty whether or not this substance was absent from the irradiated solutions. However, we were able to show that no significant amount of formylkynurenine was formed when oxindolylalanine itself was irradiated; it may, therefore, be concluded that this substance cannot act as an intermediate in this process.

Under the conditions described above, it has been shown that the presence of molecular oxygen is necessary for the formation of formylkynurenine. In general, the presence of oxygen during irradiation can have a twofold effect: (*a*) the formation of hydroperoxy radicals, following the initial process (1) viz.

$$H + O_2 \rightarrow HO_2; \qquad (2)$$

(*b*) the reaction of oxygen with an organic radical (R) to produce an organic peroxy radical (cf. Johnson, Scholes & Weiss, 1953), viz.

$$R\cdot + O_2 \rightarrow RO_2. \qquad (3)$$

It is not unlikely, therefore, that an organic hydroperoxide (RO_2H) may eventually be formed. In the case of tryptophan (I) the hydroperoxide resulting from initial attack by OH radicals at the 2–3 bond of the indole ring could decompose to give formylkynurenine (II) according to the above scheme.

Reaction *c*, i.e. conversion of the organic peroxy radical into the corresponding hydroperoxide, could result, for example, from dismutation between two peroxy radicals (cf. Johnson *et al.* 1953).

It is of interest to compare our findings with those of the biological oxidation of tryptophan. Recent experiments have shown that oxindolylalanine is not an intermediate in the *in vivo* conversion of tryptophan into formylkynurenine (Sakan & Hayaishi, 1950; Dalgliesh, Knox & Neuberger, 1951). It may be of some significance, therefore, that the irradiation of oxindolylalanine itself does not give kynurenine.

SUMMARY

1. It has been shown that formylkynurenine is one of the products formed when DL-tryptophan is irradiated with X-rays in aqueous solution in the presence of molecular oxygen.

2. It has been concluded that oxindolylalanine (α-amino-β-oxindol-3-ylpropionic acid) is not an intermediate in the reaction mechanism since irradiation of this substance itself does not lead to any significant amounts of formylkynurenine.

3. The mechanism of these processes has been discussed, with particular reference to the role played by molecular oxygen, and attention has been drawn to the similarity to the biological oxidation of tryptophan.

We would like to express our thanks to Dr A. Neuberger, F.R.S., and to Dr C. E. Dalgliesh for specimens of kynurenine, formylkynurenine and oxindolylalanine. We are indebted to the Northern Council of the British Empire Cancer Campaign for financial support.

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Transglucosidation Reactions with Flavins

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The enzymic formation of riboflavinyl glucoside by a transfer of glucose from maltose to riboflavin was described by Whitby (1952), and the reaction may be written thus:

Riboflavin + maltose →
riboflavinyl α-D-glucoside + glucose. (1)

It was concluded that a transglucosidase catalysed this reaction, and the characteristics of this enzyme, particularly with regard to its specificity, have now been investigated in an attempt to assess the physiological significance of riboflavinyl glucoside.

EXPERIMENTAL

Materials

D-Riboflavin (Roche Products, Ltd.) was used, and riboflavinyl glucoside was prepared enzymically (Whitby, 1952). D- and L-Araboflavin and the 9-substituted-*iso*alloxazines used in this work were the generous gift of Prof. P. Karrer; galactoflavin, L-lyxoflavin and *iso*riboflavin were kindly supplied by Dr M. Tishler. Flavin solutions were at all times protected from light as much as possible.

6:7-Dimethyl-9-(5′-[β-D-glucopyranosyl]-D-ribityl)-*iso*alloxazine and 6:7-dimethyl-9-(4′-[α-D-glucopyranosyl]-D-glucityl)-*iso*alloxazine have been synthesized (Plant, 1952); small amounts of these two compounds, riboflavinyl β-D-glucoside and maltoflavin, respectively, were kindly provided by Dr Plant.

α-D-Glucose 1-phosphate (dipotassium salt) was prepared by the method of Hanes (1940).

Methyl maltoside was prepared by refluxing maltose with methanolic HCl (Fischer, 1893, 1895). Other carbohydrates and derivatives were commercial samples, except for melezitose and turanose, kindly supplied by Dr D. J. Bell, and *iso*maltose octaacetate, maltulose and panose which were kindly provided by Dr W. J. Whelan.

*iso*Maltose octaacetate (15 mg.) was saponified with sodium methoxide under conditions similar to those used by Wolfrom, Georges & Miller (1949); the methanolic solution was diluted with water at 0°, immediately neutralized with HCl, and the methanol removed *in vacuo* at 35°. The solid residue was dissolved in water and the solution used for enzymic tests; it showed only one spot on chromatograms which had been developed with butanol:acetic acid:water (4:1:5, v/v/v) and sprayed with aniline hydrogen phthalate (Partridge, 1949), the spot having $R_F = 0{\cdot}10$, compared with 0·12 for maltose.

Phosphate buffers were prepared with K_2HPO_4 and NaH_2PO_4, $2H_2O$.

Enzyme preparation. An acetone powder of rat liver was made by the method of Whitby (1952), and the following manipulations were all carried out in a cold room at 2°. The powder (40 g.) was extracted for 20 min. in a mortar with 400 ml. of 0·1 M phosphate buffer, pH 7·4, and the suspension centrifuged for 15 min. at 1800 *g*; the supernatant was collected, the centrifugate extracted a second time with 400 ml. of buffer for 20 min., centrifuged, the supernatants combined (728 ml.) and the solid residue discarded. M-Phosphate buffer (182 ml.), pH 7·4, and 131 g. of $(NH_4)_2SO_4$ were added. After 30 min. the precipitate was collected by centrifuging at 1800 *g* for 10 min. and was discarded. $(NH_4)_2SO_4$ was added to increase the saturation from 0·25 to 0·55 and the precipitate was collected 1 hr. later by centrifuging for 5 min. at 15000 *g* (Servall Centrifuge); the supernatant was discarded. The precipitate was dissolved in 50 ml. of 0·05 M phosphate buffer, pH 7·4, and dialysed overnight with stirring against 4 l. of 0·05 M phosphate buffer, pH 7·4. The dialysed solution was frozen at −20° and dried *in vacuo* over H_2SO_4 and NaOH. The powder weighed 7·3 g. and is henceforth referred to as 'the enzyme'; its activity did not deteriorate over 6 months when stored *in vacuo* over $CaCl_2$. Attempts at further purification of the enzyme by preferential thermal inactivation of contaminating protein, with and without the addition of maltose to protect the enzyme, by adsorption and elution from calcium phosphate gel, by precipitation below pH 5·5, etc., have not been successful; however, this impure enzyme has proved to be satisfactory for the following experiments.

Methods

The pH was measured with a Cambridge pH meter (Cambridge Instrument Co.). The instrument was equilibrated at 35° for 2 days before determining the pH/activity relationship for synthesis of riboflavinyl glucoside.

Spectrophotometric measurements were made in quartz cells, 1 cm. thickness, in a Beckman model DU spectrophotometer. Flavins were estimated in 0·1 M phosphate buffer, pH 7·4. Solutions of all the 6:7-dimethyl-9-substituted-*iso*alloxazines investigated here, e.g. riboflavin and galactoflavin, were estimated by their light absorption at 450 mμ., using the molecular extinction coefficient (ϵ) = $12{\cdot}2 \times 10^3$ l. mole^{-1} cm.$^{-1}$ (Singer & Kearney, 1950*a*; Whitby, 1952), because of the close similarity of their absorption spectra. *iso*Riboflavin was estimated at 450 mμ., using $\epsilon = 7{\cdot}3 \times 10^3$ l. mole^{-1} cm.$^{-1}$ (Tishler, Pfister, Babson, Ladenburg & Fleming, 1947). The three 9-substituted-*iso*alloxazines had similar spectra, with a maximum at 435 mμ., which was used for their estimation. The value of ϵ at 435 mμ. was determined with a sample of 9-(L-1′-arabityl)-*iso*alloxazine which had been dried *in vacuo* at 100° over P_2O_5 for 1 hr., and $\epsilon = 10{\cdot}4 \times 10^3$ l. mole^{-1} cm.$^{-1}$ was observed. (Found: C, 50·7; H, 4·6; N, 20·1. Calc. for $C_{15}H_{16}O_6N_4$: C, 51·7; H, 5·2; N, 20·1 %.) Kuhn & Weygand (1934) observed that 9-methyl-*iso*alloxazine had an absorption maximum at 440 mμ., and from their spectrum $\epsilon \simeq 9{\cdot}4 \times 10^3$ l. mole^{-1} cm.$^{-1}$ at this wavelength.

Fluorimetric measurements were made with a fluorimetric attachment supplied for use with Beckman spectrophotometers. Measurements were made in 0·1 M phosphate buffer, pH 7·4, and standard curves were obtained with riboflavin and with 9-(L-1′-arabityl)-*iso*alloxazine for estimating the glucosides of 6:7-dimethyl-9-substituted-*iso*alloxazines (e.g. riboflavinyl glucoside) and of 9-substituted-*iso*alloxazines, respectively; a separate standard curve was made with *iso*riboflavin. The curves were linear up to a concentration of 1 μg. of flavin/ml., and the fluorescence of riboflavinyl glucoside was found to be quantitatively equivalent to that of riboflavin under these conditions.

Estimation of enzymic activity. The contents of each incubation mixture are detailed below, but the preparation of the chromatograms and the estimation of flavin glucoside were performed similarly in each case. The estimation of the Michaelis constant for riboflavin in the synthesis of riboflavinyl glucoside from riboflavin and maltose is described here to illustrate the procedure.

Enzyme (160 mg.) was dissolved in 4 ml. of 0·1 M maltose and 4 ml. of M phosphate buffer, pH 7·4, and kept at 0°; 1 ml. of this solution was added to tubes containing 0·5 ml. of riboflavin solution of different strengths (prepared by dilution of a supersaturated (1·94 mM) solution of riboflavin) and the solutions incubated in darkness at 35°. Sheets of Whatman no. 3 filter paper (57 × 46 cm.) had been prepared, and 0·1 ml. samples of each incubation mixture were applied along a line ruled parallel to the longer side of the paper, 8 cm. distant from its edge. Samples were applied with the minimum exposure to direct light and, to minimize the area of each spot as well as to reduce the time needed for application, the paper was heated during the application of a sample by pressing against glass tubing through which water was syphoning from a bath of boiling water. The spots were approximately 4 cm. wide and 1·5 cm. deep and the centres of spots were 8 cm. apart; samples were taken in triplicate at 0, 1, 2 and 3 hr. (except where otherwise stated) and, immediately after the application of samples at each of these times, the papers were heated in an oven at 105° for 5 min. to denature the enzyme. The papers were equilibrated with solvent vapour (water, saturated with *iso*amyl alcohol) for 6 hr. and then run as ascending chromatograms for 12 hr.

After development, chromatograms were dried in darkness at 37°; it was essential to hang the chromatograms at right-angles to the direction of solvent development, as otherwise the separation of flavin glucoside from unchanged flavin was sometimes impaired by migration of spots during the drying process. The chromatograms were examined by ultraviolet light and the areas containing flavin glucoside marked out with pencil. Riboflavinyl glucoside has $R_F = 0{\cdot}5$ and riboflavin $R_F = 0{\cdot}4$ in this solvent; other flavin glucosides are separated similarly from their parent flavins. Each spot was cut out and extracted with 4 ml. of 0·1 M phosphate buffer, pH 7·4, by heating in glass-stoppered tubes for 10 min. in darkness at 60–70°. The solutions were cooled and most of the filter paper was removed with a forceps; fine particles of filter paper were removed by centrifuging, and the flavin glucoside was then estimated in the fluorimeter. Recovery of standard riboflavin solutions, applied to filter paper and eluted as described above, was 95–98 %. Excessive exposure to light during the early manipulations led to the appearance of lumichrome, a blue-fluorescent spot having $R_F = 0{\cdot}1$, on the chromatograms.

In experiments on flavin specificity, where sufficient flavin was added to saturate the solutions, samples of each incubation mixture were cooled rapidly to 0° and centrifuged at 0° before being applied to filter paper. This avoided the excessive trailing which occurs whenever undissolved flavins are applied to chromatograms.

In the experiments on carbohydrate specificity, a control determination was made with riboflavin present, but with no maltose nor other carbohydrate added, since the enzyme preparation contained a small amount of glucose donor from which riboflavinyl glucoside was synthesized in minute amount, and a correction could thus be applied.

RESULTS

Quantitative characteristics of the transglucosidase

Riboflavin. The experimental details are described in the previous section, and the results were plotted by the method of Lineweaver & Burk (1934). The Michaelis constant for riboflavin at 35° and pH 7·4 in reaction (1) was found to be 0·41 mM.

Maltose. 0·05 M Maltose was prepared in a supersaturated solution of riboflavin (2·75 mM in 0·5 M phosphate buffer, pH 7·4) and a series of dilutions of this maltose solution made by dilution with the riboflavin solution. The enzyme was also dissolved in more of this supersaturated solution of riboflavin in order that the Michaelis constant for maltose might be determined in the presence of a high concentration of riboflavin. The Michaelis constant for maltose at 35° and pH 7·4 in reaction (1) was found to be 0·82 mM.

α-D-*Glucose* 1-*phosphate.* The previous experiment was repeated in the presence of α-glucose 1-phosphate (1·50 mM) and the results are shown in Fig. 1. The inhibition of reaction (1) by α-glucose 1-phosphate is competitive, and the inhibitor constant at 35° and pH 7·4 is 1·7 mM.

pH/*activity relationship* was investigated between pH 5·7 and 8·5; phosphate and pyrophosphate buffers were used; below pH 5·5 the enzyme is unstable. Each tube contained 0·6 ml. of buffer, 0·15 ml. of 0·05 M maltose and 0·75 ml. of enzyme (160 mg. dissolved in 6 ml. of 1·92 mM riboflavin). Fig. 2 shows that the optimum synthesis of riboflavinyl glucoside from maltose and riboflavin is obtained at pH 6·7.

Glucosidase activity of the enzyme preparation

Breakdown of riboflavinyl glucoside. This was studied in the presence and in the absence of glucose, in order to differentiate between reaction mechanisms (2) and (3), as explained in the Discussion.

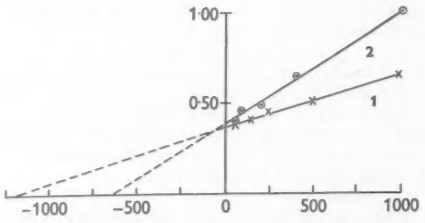

Fig. 1. Inhibition of the synthesis of riboflavinyl glucoside by α-D-glucose 1-phosphate. Curve 1, glucose 1-phosphate absent; curve 2, 1·50 mM glucose 1-phosphate present. Ordinate: reciprocal of rate of formation of riboflavinyl glucoside [1/(μg./mg. enzyme/hr.)]; abscissa: reciprocal of molar concentration of maltose.

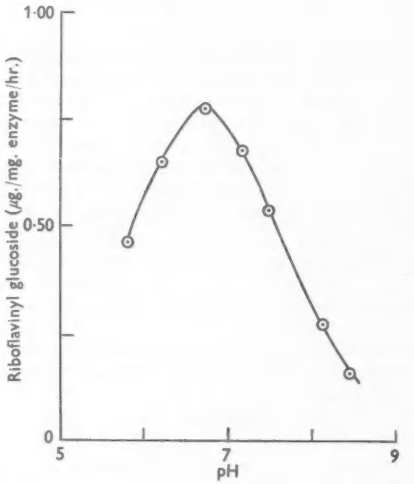

Fig. 2. The effect of pH on the rate of synthesis of riboflavinyl glucoside.

Enzyme (50 mg.) was dissolved in 2·5 ml. of 0·1 M phosphate buffer, pH 7·4, and incubated in a Warburg manometer with 0·2 ml. of 0·1% notatin. A small uptake of O_2 (15 μl.) occurred during the first 5 min. after mixing the manometer contents, but no further uptake was observed during the next 30 min. Enzymically prepared riboflavinyl glucoside (1·5 ml. of 2·46 mM solution, dissolved in 0·1 M phosphate buffer, pH 7·4) was incubated in a manometer with 0·2 ml. of notatin at 38°; no gas exchange occurred.

Enzyme solution (0·65 ml.) and 0·25 ml. of riboflavinyl glucoside solution were removed from the manometers and added to a tube containing 0·1 ml. of M glucose solution; similar amounts of enzyme and of riboflavinyl glucoside were added to a tube containing 0·1 ml. of water. The tubes were incubated at 35° and duplicate samples taken from each at 0, 0·5, 1 and 1·5 hr. Riboflavin was separated chromatographically from riboflavinyl glucoside and estimated fluorimetrically; no riboflavin was present in the samples taken at 0 hr. Riboflavin was formed both in the presence and absence of 0·1 M glucose, and the rate of formation of riboflavin was equal in the two experiments. It was confirmed by manometric measurement that the notatin had not completely oxidized the added glucose after 2 hr. at 35°.

The Michaelis constant for riboflavinyl glucoside in its breakdown to riboflavin was determined. Enzyme (80 mg.) was dissolved in 1·5 ml. of M phosphate buffer, pH 7·4, and 0·25 ml. added to tubes containing 0·75 ml. of riboflavinyl glucoside solution of different strengths; duplicate samples were taken at 0, 0·5, 1 and 1·5 hr. The Michaelis constant for riboflavinyl glucoside at 35° and pH 7·4 was found to be 0·18 mM.

Action on other glycosides. 0·025 M Solutions of maltose, cellobiose, lactose and α-methyl glucoside were incubated manometrically (total volume = 2·2 ml.) in 0·1 M phosphate buffer, pH 7·4, at 38° in the presence of enzyme (13 mg./ml.) and notatin (0·1 mg./ml.). With maltose, a steady uptake of oxygen occurred (104 μl./hr.), but with the other sugars no uptake occurred after a small initial uptake of 8–12 μl. in each case; the latter figure is approximately equivalent to the small reaction observed in the previous experiment where notatin was used to free the enzyme from glucose or glucose-forming substances. Control experiments showed that the notatin did not exhibit any maltase activity (cf. Whistler, Hough & Hylin, 1953).

Riboflavinyl β-D-glucoside, maltoflavin and enzymically prepared riboflavinyl glucoside were each added in sufficient amount to saturate 0·3 ml. of enzyme solution (4 mg. enzyme in 0·1 M phosphate buffer, pH 7·4) and the three solutions incubated at 35° (the strength of each flavin solution was approximately 6 mM). Samples (50 μl. each) were taken at 0, 1·5, 3 and 4·5 hr., and the chromatograms developed with butanol:acetic acid:water (4:1:5, v/v). No formation of riboflavin was observed from riboflavinyl β-D-glucoside, but with maltoflavin ($R_F = 0·18$) as substrate a second fluorescent spot was detected ($R_F = 0·26$). This spot

was assumed to be glucoflavin and its intensity increased during the incubation. No sample of glucoflavin was available, but the isomeric galactoflavin has $R_F = 0{\cdot}26$ in this solvent. No accurate comparison of the rates of breakdown of maltoflavin and of enzymically prepared riboflavinyl glucoside could be made, because of the small amount of maltoflavin available, but it was apparent that maltoflavin was being broken down much more slowly than enzymically prepared riboflavinyl glucoside; glucoflavin was formed from maltoflavin at less than 5% of the rate of formation of riboflavin, as judged by comparison of the intensities of fluorescence of their spots on the chromatograms.

Synthesis of glucosides from various iso*alloxazine derivatives*

Enzyme (420 mg.) was dissolved in 1·4 ml. of M phosphate buffer, pH 7·4, 7 ml. of 0·05M maltose and 5·6 ml. of water; 1·5 ml. of this solution was saturated with each of the flavins listed in the first column of Table 1. The experiment was performed at one concentration only with each flavin, because of shortage of these substrates, except for riboflavin where the Michaelis constants have been determined for reaction (1). Except with 9-oxyethyl-*iso*alloxazine, a second spot appeared on the chromatograms, and the rate of synthesis of this second spot is given in the third column of Table 1. The last column of Table 1 relates the rate of flavin glucoside synthesis to the concentration of the parent flavin.

The proof that the synthesis of a flavin glucoside is being measured in this experiment rests upon the following facts. If D-araboflavin, for instance, is incubated under these conditions without added maltose, the formation of only a minute amount of a second flavin can be detected on paper chromatograms, but the amount of this second flavin is greatly increased if maltose is added to the reaction mixture. D-Araboflavin is stable in the presence of maltose if the enzyme has been previously inactivated by being boiled for 5 min., and no second spot can then be detected on chromatograms. The relative positions of D-riboflavin and riboflavinyl glucoside (for R_F values, see Whitby, 1952) are similar to the relative positions of D-araboflavin and its derived flavin when chromatographed in the same solvents (e.g. in butanol:acetic acid:water (4:1:5, v/v) and in water, saturated with *iso*amyl alcohol, D-araboflavin has R_F values 0·30 and 0·52, and its derived flavin has 0·20 and 0·60, respectively). D-Araboflavinyl glucoside has been made on a small scale, hydrolysed and the liberation of glucose demonstrated chromatographically (see below). Only in the case of riboflavinyl glucoside have the position and configuration of the glucosidic link been determined (Whitby, 1952); by analogy, it may be inferred that glucose is transferred from maltose to the terminal position of the polyhydroxylic side chains of the flavins studied in Table 1, to give an α-glucoside.

D-*Araboflavinyl glucoside.* Enzyme solution (2 ml.; 60 mg. of enzyme and 0·05M maltose in 0·2M phosphate buffer, pH 7·4) was saturated with D-araboflavin and incubated at 35° for 5 hr.; undissolved D-araboflavin was removed by centrifuging and the solution applied to a sheet of Whatman no. 3 paper (a blank sheet of paper was similarly treated in the subsequent steps). The enzyme was denatured, the chromatogram developed with water, saturated with *iso*amyl alcohol, and the faster-moving area ($R_F = 0{\cdot}6$) was cut out, eluted with water at 70° and the solution evaporated to dryness. The residue was chromatographed a second time to ensure that all glucose and maltose had been removed (they ran near the solvent front) and, after elution with 10 ml. of water, the flavin solution was refluxed with an equal volume of NH_3 (sp.gr. 0·880) at 100° for 1 hr. to destroy the alkali-labile ring of the *iso*alloxazine nucleus (Kuhn & Rudy, 1934); treatment with NH_3 eliminated the intense yellow colour of flavin, which would otherwise have interfered with the chromatographic detection of glucose. Ammonia was removed *in vacuo* and the solid residue heated with 2 ml. of 2N-HCl at 100° for 2 hr.; the solution was cooled, passed through a column of Amberlite 1R-4B (acetate form), frozen at −20° and dried over P_2O_5 and

Table 1. *Synthesis of glucosides of various* iso*alloxazine derivatives*

In these reactions maltose was the glucose-donating substrate. For conditions, see the text.

Glucose-accepting substrate	Concentration (M × 10^4) *A*	Glucoside synthesized (mole × 10^9/hr.)/mg. enzyme *B*	Expressed as percentage of the action with riboflavin *B*/*A*
D-Riboflavin	5·3	1·08	100
L-Araboflavin	6·8	1·30	94
D-Araboflavin	5·5	1·10	98
L-Lyxoflavin	0·55	0·11	98
D-Galactoflavin	7·0	0·02	1–2
*iso*Riboflavin	2·8	0·37	65
9-L-Arabityl-*iso*alloxazine	12·0	2·25	92
9-Dihydroxypropyl-*iso*alloxazine	2·5	0·16	31
9-Oxyethyl-*iso*alloxazine	2·1	0	0

NaOH. The residue was chromatographed in butanol:acetic acid:water (4:1:5, v/v) and the chromatogram sprayed with aniline hydrogen phthalate (Partridge, 1949); glucose was detected, but nothing was found in the blank, which had been treated similarly throughout in case Whatman no. 3 filter paper had yielded some glucogenic material under these conditions.

Enzymic synthesis of riboflavinyl glucoside from riboflavin and various glucose donors

The following sugars (0·03 M) were incubated at pH 7·4 (0·1 M phosphate buffer) with enzyme (13·3 mg./ml.) and riboflavin (1·72 mM) and found to be unable to replace maltose as glucose donor in reaction (1): α-D-glucose 1-phosphate, α-methyl glucoside, cellobiose, gentiobiose, *iso*maltose, lactose, melezitose, melibiose, methyl maltoside, panose, raffinose, sucrose and αα-trehalose. The competitive inhibition of reaction (1) by α-glucose 1-phosphate has already been described. Under identical conditions it was found that maltulose (4-*o*-α-glucopyranosyl-D-fructose) and turanose (3-*o*-α-glucopyranosyl-D-fructose) were able to replace maltose in reaction (1), the relative rates of synthesis of riboflavinyl glucoside from maltose, maltulose and turanose being 10:9:6. The proof that riboflavinyl glucoside has been synthesized from riboflavin and maltulose or turanose rests on evidence similar to that advanced in the case of the flavin glucosides listed in Table 1. Riboflavinyl glucoside was prepared enzymically from riboflavin and turanose, degraded in the manner described for D-araboflavinyl glucoside, and the liberation of glucose detected chromatographically.

Solubility of riboflavinyl glucoside

Riboflavinyl glucoside was shaken with 0·1 M phosphate buffer, pH 7·4, under toluene for 2 days at 20 and 37°. The solutions were centrifuged to remove undissolved riboflavinyl glucoside, and the strength of each solution was determined. Riboflavinyl glucoside was shown to be stable under these conditions, and neither riboflavin, lumiflavin nor lumichrome could be detected chromatographically. The solubilities at 20 and 37° were 2·2 and 3·5 mg./ml., respectively, which should be compared with the corresponding solubilities of riboflavin of 0·1 and 0·2 mg./ml., respectively, under these conditions.

DISCUSSION

The glucosidic link in enzymically synthesized riboflavinyl glucoside was not definitely shown to be an α-linkage by Whitby (1952), but the fact that the glucosidase investigated here was unable to hydrolyse synthetic riboflavinyl glucoside, which is believed to be a β-glucoside (Plant, 1952), coupled with its ability to hydrolyse the enzymically synthesized compound under identical conditions, is further evidence for the latter being riboflavinyl α-D-glucoside.

The breakdown of riboflavinyl α-D-glucoside might proceed by either or both of the following routes:

$$\text{Riboflavinyl α-D-glucoside} + \text{glucose} \rightarrow \text{riboflavin} + \text{maltose}, \quad (2a)$$

$$\text{Maltose} + \text{water} \rightarrow 2\ \text{glucose}, \quad (2b)$$

$$\text{Riboflavinyl α-D-glucoside} + \text{water} \rightarrow \text{riboflavin} + \text{glucose}. \quad (3)$$

Reaction (2*a*) would be catalysed by a transglucosidase and would require the presence of glucose to initiate it, whereas reaction (3) would be catalysed by a glucosidase. The enzyme preparation was shown to catalyse reaction (2*b*) so, to differentiate between reactions (2) and (3), it was necessary to remove glucose and glucose-forming substances from one set of solutions. This was effected by previous incubation with notatin, and the presence of notatin during the experiment would also have prevented reaction (2*a*) from becoming autocatalytic. The rate of breakdown of riboflavinyl α-D-glucoside was not reduced in the absence of glucose; from this it could be deduced that reaction (2*a*) was not obligatory for the breakdown of riboflavinyl α-D-glucoside, that reaction (3) took place, and that in all probability two different enzymes were not present (one to catalyse reaction (2*a*) and the other reaction (3)) since the rate of breakdown would then have been expected to be greater in the presence of glucose than in its absence. The experiment does not distinguish between the following possibilities: that two different enzymes catalyse the synthesis and the breakdown of riboflavinyl α-D-glucoside (a transglucosidase and a glucosidase respectively); that there is a single enzyme which has both transglucosidase and glucosidase activities. The latter possibility is favoured because of similarities in specificity between the transglucosidase and glucosidase activities of the enzyme preparation. Bealing & Bacon (1953) believe that the β-fructofuranosidase of *Penicillium spinulosum* has both transferase and hydrolase activities.

The transglucosidase will not synthesize a glucoside of 9-oxyethyl-*iso*alloxazine, which is a monohydric alcohol, nor will the glucosidase hydrolyse α-methyl glucoside, in which the aglycone is a monohydric alcohol. Secondly, Table 1 shows that the rate of synthesis of galactoflavinyl glucoside is slow relative to the rate of synthesis of riboflavinyl α-D-glucoside, and it was found that the glucosidase hydrolysed maltoflavin slowly when compared with riboflavinyl α-D-glucoside; these two experiments, although not entirely comparable,

since galactoflavinyl glucoside is thought to be the 6′-glucoside, whereas maltoflavin is the 4′-glucoside, do suggest that the pentityl side chain of riboflavin is more suited structurally to both glucosidase and transglucosidase activity than the hexityl side chains of galactoflavin and glucoflavin.

Table 1 shows that the transglucosidase is able to synthesize glucosides of several *iso*alloxazine derivatives, but the relative affinity of the enzyme for the different compounds cannot be deduced from the table because the comparison was made in terms of concentrations and not Michaelis constants. The low reactivity of galactoflavin is noteworthy because yeast flavokinase does not phosphorylate galactoflavin (Kearney, 1952) and because galactoflavin is an antivitamin (Emerson, Wurz & Johnson, 1945).

Maltose can be replaced by maltulose or turanose in reaction (1), but the relative affinity of the enzyme for these three disaccharides has not been assessed in terms of their Michaelis constants. The specificity requirements of the enzyme for the carbohydrate donor can be partly defined by considering the points of structure common to maltose, maltulose and turanose, and contrasting them with the many compounds which have been found to be unable to replace maltose in reaction (1). Only reducing disaccharides having an α-glucosidic link have been found to be active, and the inability of *iso*maltose and panose to replace maltose shows that the point of linkage between the monosaccharides is important. D-Glucose and D-fructose can function as aglycone, and possibly other compounds may do so too; only α-glucopyranosides have been found active (melibiose, an α-galactoside, was inactive, but it has the 1:6-linkage, like *iso*maltose), so the enzyme may be a transglycosidase or, more specifically, a transglucosidase.

Yeast invertase, acting on sucrose in the presence of methanol, was shown to produce methyl β-fructofuranoside (Bacon, 1952), and the formation of possibly analogous compounds was observed from three other monohydric alcohols. Bealing (1953) incubated mould extracts with sucrose or raffinose and various monohydric and dihydric alcohols, and observed the appearance of new fructose-containing spots on chromatograms; these were thought to be the corresponding alkyl β-fructofuranosides. Flavin glucosides are formed by a similar reaction from maltose and certain polyhydric *iso*alloxazines, but the enzyme differs in its specificity requirements from the enzymes studied by Bacon (1952) and by Bealing (1953) since it does not transfer glucose to the monohydric alcohol, 9-oxyethyl-*iso*alloxazine. It is possible that the proximity of the heterocyclic structure to the alcoholic group inhibited the enzyme completely, but this would not explain the rate of synthesis of galactoflavinyl glucoside, which is very slow compared to the rate of synthesis of other flavin glucosides (Table 1). The enzyme which catalyses the synthesis of flavin glucosides must require other conditions to be fulfilled by the acceptor molecule besides the possession of a primary alcoholic group. Another difference, possibly, between this transglucosidase and the transfructosidases described by Bacon (1952) and Bealing (1953) is that three reducing disaccharides have been found to act as glucose donors with the former enzyme and three non-reducing disaccharides have been found to be inactive, whereas the non-reducing sugars sucrose or raffinose are required for the transfructosidations (no reducing disaccharide containing β-fructofuranose has been tested).

If the enzyme catalysing reaction (1) had been found to have an absolute specificity for D-riboflavin, it would have been possible to infer that riboflavinyl α-D-glucoside had a physiological function, but Table 1 shows that glucosides of antivitamins, such as D-araboflavin (Euler & Karrer, 1946) and *iso*riboflavin (Emerson & Tishler, 1943), can also be synthesized enzymically. However, yeast flavokinase phosphorylates D-araboflavin and dichloroflavin in addition to D-riboflavin (Kearney, 1952), and *iso*riboflavin is more effective in catalysing the non-enzymic reduction of cytochrome *c* by reduced pyridine nucleotides than the flavins which are known to occur naturally (Singer & Kearney, 1950*b*), so the enzymic synthesis of glucosides from antagonists of vitamin B_2 does not necessarily mean that riboflavinyl α-D-glucoside has no physiological significance. It is much more soluble in water than riboflavin and this property may be of importance in the transport of, perhaps, the least water-soluble of all the water-soluble vitamins. This suggestion would be more acceptable if riboflavinyl α-D-glucoside had been shown to occur naturally; it has not been observed in tissue extracts, but it may have been hydrolysed enzymically to riboflavin in the early stages of the isolation procedures adopted. Bessey, Lowry & Love (1949) estimated the amount of different flavins in various tissues and found that flavin mononucleotide and flavin-adenine dinucleotide comprised the major part of the total flavin. If riboflavinyl α-D-glucoside forms part of the unphosphorylated flavin in tissues, it can only be present in small amount, which may explain why it has not been detected.

SUMMARY

1. The specificity of the enzyme which synthesizes riboflavinyl glucoside has been investigated in an attempt to assess the physiological significance of this derivative of riboflavin. It is possible that the

enzyme has both transglucosidase and glucosidase activities.

2. The transglucosidase synthesizes glucosides of several *iso*alloxazine derivatives, many of which are antivitamins. Certain specificity requirements have been defined for the glucose donor and for the flavin acceptor.

3. The quantitative characteristics of the formation of riboflavinyl glucoside from riboflavin and maltose have been investigated, and the inhibition of this reaction by α-D-glucose 1-phosphate has been shown to be competitive.

4. The solubility of riboflavinyl glucoside in water is much greater than the solubility of riboflavin, and it is suggested that this may be of importance in the transport of riboflavin.

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Comparative Detoxication

3. HIPPURIC ACID FORMATION IN ADULT LOCUSTS

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It has been shown that the detoxication of phenols in the locust is accomplished by a conjugation mechanism different from that used by vertebrates, glucosides rather than glucuronides being formed (Myers & Smith, 1954). It was of interest, therefore, to consider the fate of aromatic acids in this insect, since even among vertebrates much variation is found in the method of conjugation of these acids, which may be condensed with glucuronic acid, glycine, ornithine, or glutamine in different species (Williams, 1947). Previous work has given very little indication of the existence of a conjugation mechanism in insects for the detoxication of aromatic acids though the reported presence of hippuric acid in some insects suggested that this might be possible. This identification of hippuric acid, in the faeces of various caterpillars (Davy, 1854; van der Hoeven, 1850), and in the fat body of *Dytiscus* (Sirodt, 1858) rests on microscopical observation of crystal form and is uncertain.

The presence of some conjugated benzoic acid such as hippuric acid in the excreta of locusts, which feed on grass, might be expected since this compound is a typical urinary constituent of vertebrate herbivores. However, Brown (1937) was unable to find hippuric acid in the excreta of another Acridid, the grasshopper, *Melanoplus bivittatus*.

We have studied this detoxication mechanism by observing the metabolism of some aromatic acids whose glycine conjugates can readily be detected in the excreta, and it will be shown that the locust, like most animals, conjugates the aromatic acids with glycine to form hippuric acids.

EXPERIMENTAL

Reference compounds. *p*-Nitrohippuric acid, m.p. 135°, and dibenzoylornithine (ornithuric acid), m.p. 185°, were prepared by the Schotten-Baumann reaction using the appropriate amino acids and aroyl chlorides. *o*-Hydroxyhippuric acid (salicyluric acid), m.p. 166°, was prepared from salicylic azide according to Bondi (1907). Hippuric acid, m.p. 186°, and *p*-aminohippuric acid, m.p. 199°, were com-

mercial products. The diphenylmethyl ester of hippuric acid was prepared from diphenyldiazomethane and hippuric acid (cf. Elvidge, Linstead, Sims & Orkin, 1950). *Diphenylmethyl hippurate* formed narrow plates, m.p. 123–124°. (Found: C, 76·1; H, 5·0. $C_{22}H_{19}NO_3$ requires C, 76·5; H, 5·6%.)

Locusts. Adult *Locusta migratoria*, obtained from the Anti-Locust Research Centre, London, were maintained on a liberal diet of fresh grass. Compounds were administered mixed with crushed biscuit or by injection with an Agla micrometer syringe (Burroughs Wellcome Ltd.). In quantitative experiments separate insects were kept in 500 ml. beakers on a restricted grass diet (cf. Myers & Smith, 1953, 1954).

Preparation of extracts. The droppings were roughly ground to break up the pellets and boiled with water (about 10 ml./g. of faeces). This extract was filtered with suction and the filtrate evaporated to dryness at reduced pressure. The residue was dissolved in hot 80% ethanol (10 ml./g.), filtered and the filtrate concentrated to small bulk. This concentrate was then submitted to a preliminary separation on a large-scale paper chromatogram (cf. Myers & Smith, 1954) and any metabolites were located by suitable colour tests. Elution of appropriate sections of this chromatogram gave extracts suitable for chromatographic or spectrophotometric characterization.

Paper chromatography. This was carried out as described by Smith, Smithies & Williams (1953) and the R_F values obtained are quoted in Table 1. Unless stated otherwise, extracts were compared with reference compounds in all of the solvents in this table. The preparation and chromatography of the dinitrophenyl derivatives of the amino acids were carried out as described by Krol (1952) except that Whatman no. 1 paper, prepared by soaking in the buffer and drying, was used instead of a Celite column. The dried, buffered papers were then used in the normal way.

Detection of acids. The acids were conveniently located on the papers by illumination with ultraviolet light from a Hanovia Chromatolite lamp which emits mainly radiation of 254 mμ. wavelength. In this light, salicylic acid and salicyluric acid fluoresced blue, *p*-aminobenzoic and *p*-aminohippuric acids feebly purple. *p*-Nitrobenzoic and *p*-nitrohippuric acids strongly quenched the fluorescence of the paper and appeared as dark spots. Benzoic, hippuric and ornithuric acids could also be detected by a weak quenching effect. These three acids were also located as blue spots when the paper, after drying at room temperature for 24 hr., was sprayed with 2% (w/v) starch solution containing 5% (w/v) each of KIO_3 and KI. Aromatic amino compounds were located by diazotization and coupling with β-naphthol. Glycine was detected by the *o*-phthalaldehyde reagent (Sandstrom & Lillevik, 1941) which gave a green colour, and other amino acids by heating the paper at 100° after spraying with 0·1% ninhydrin in butanol.

Ultraviolet absorption spectra. These were measured in a Unicam S.P. 500 Spectrophotometer.

Estimation of amino compounds in faeces. The droppings from each locust were broken up with a glass rod under 2 ml. of 2 N-NH_3 in a 10 ml. centrifuge tube and mixed with 3 ml. of ethanol. After centrifuging down the debris, 1 ml. of the clear supernatant liquid was evaporated in a current of warm air on Whatman no. 4 paper to give a spot about 2 cm. diameter, 10 cm. from the end of the paper. The paper was irrigated with a saturated solution of water in ethyl methyl ketone for 4–5 hr. and dried. The zone of the chromatogram containing *p*-aminohippuric acid was located by spraying a reference spot with the diazo reagents and corresponding strips of paper cut out and eluted chromatographically with 2–3 ml. of 0·1 N-NH_3. *p*-Aminohippuric acid was estimated in this eluate by the colorimetric method of Bratton & Marshall (1939) using *N*-1-naphthylethylenediamine as a coupling agent. A standard curve was constructed using *p*-aminohippuric acid, and colour intensities were measured in a Spekker Absorptiometer with an Ilford 604 filter (maximum transmission, 520 mμ.).

Recoveries of *p*-aminohippuric acid (50–500 μg.) added to locust faeces ranged from 91 to 97% when estimated according to this technique.

Estimation of diazotizable amines were also made on the ethanolic extract before chromatography. This value represented the sum of the unacetylated aromatic amino acids excreted, since within the limits of the method *p*-aminobenzoic and *p*-aminohippuric acids gave equivalent colour intensities.

Metabolic experiments

Benzoic acid. Seventy adult locusts were allowed to feed for a week on a mixture of wet biscuit (cream crackers) which contained 5% (w/w) of benzoic acid. The faeces collected

Table 1. *R_F values of some acids and their conjugates*

Whatman no. 4 paper, run till front had moved about 30 cm.

Solvent system:

I Benzene:*n*-butanol:ammonia, sp.gr. 0·88 (2:5:2, v/v) (upper phase).
II *n*-Butanol:acetic acid:water (4:1:5, v/v) (upper phase).
III Benzene:acetic acid:water (1:1:2, v/v) (upper phase).
IV *n*-Butanol saturated with water.
V Ethyl methyl ketone saturated with water.

Acid	R_F values				
	I	II	III	IV	V
Benzoic	0·30	0·90	0·95	0·95	0·95
Hippuric	0·20	0·85	0·15	0·70	0·35
Ornithuric	0·60	0·95	0·30	0·90	0·50
p-Nitrobenzoic	0·30	0·95	0·80	0·70	0·50
p-Nitrohippuric	0·20	0·85	0·15	0·40	0·20
p-Aminobenzoic	0·05	0·95	0·80	0·70	0·85
p-Aminohippuric	0·05	0·65	0·05	0·10	0·10
Salicylic	0·40	0·90	0·90	0·60	0·45
Salicyluric	0·05	0·80	0·50	0·35	0·35

over this period were extracted with water and then ethanol, and at this stage drops of the ethanolic solution were chromatographed in the solvents shown in Table 1. The extract gave intense spots corresponding to hippuric acid and benzoic acid, but no evidence of the presence of ornithuric acid was found.

On evaporation, the ethanolic solution deposited a dark brown crystalline solid which was recrystallized several times from water (charcoal) to give colourless plates (15 mg.) of hippuric acid, m.p. and mixed m.p. 185°. (Found: C, 60·3; H, 5·5. Calc. for $C_9H_9NO_3$, C, 60·3; H, 5·1%.) These depressed the melting point of ornithuric acid. The diphenyl methyl ester of hippuric acid prepared from 5 mg. of this material had m.p. and mixed m.p. 123°.

Similar experiments with injected benzoic acid also yielded faecal extracts in which hippuric acid could be detected after paper chromatography.

No hippuric acid was found in similar extracts of the faeces of stock locusts maintained on a grass diet with no added benzoic acid.

p-*Nitrobenzoic acid.* Twelve immature adult locusts were each injected with 1 mg. of the sodium salt of the acid and the faeces collected for the next 24 hr. The crude ethanolic extract was separated in a large-scale chromatogram on no. 4 Whatman paper with benzene:acetic acid:water (1:1:2, v/v) till the front had moved 35 cm. Strips cut from the edge of the paper were then examined for metabolites under ultraviolet light and with the diazo reagents. Four bands were present, of which *A* (R_F about 0·2) and *B* (R_F about 0·9) quenched the fluorescence of the paper in ultraviolet light and two, *C* (R_F 0·05) and *D* (R_F 0·6), gave a positive diazo reaction. These areas of the paper were eluted with water and small portions of the eluates run on new chromatograms alongside reference spots in the three solvent mixtures I, II, III (Table 1). The eluate of zone *A* had the same R_F values as *p*-nitrohippuric acid, and that of zone *B* the same as *p*-nitrobenzoic acid. The extract of zone *D* was identified chromatographically with *p*-aminobenzoic acid and *C* with *p*-aminohippuric acid. From the relative intensity of the reactions it appeared that *p*-nitrobenzoic acid and *p*-aminobenzoic acid were present in larger amounts than the other two metabolites.

The bulk of the eluate of zone *A* was rechromatographed in butanol:acetic acid:water (4:1:5, v/v) and the appropriate zone eluted with water; its ultraviolet spectra in acid $\lambda_{max.}$ 268 mμ. and alkali $\lambda_{max.}$ 271 mμ., were found to be identical with those of authentic *p*-nitrohippuric acid over a range of 260–350 mμ. (Fig. 1). A strip of paper of the same size was cut from the chromatogram and eluted with water for use as a blank in the spectrophotometer.

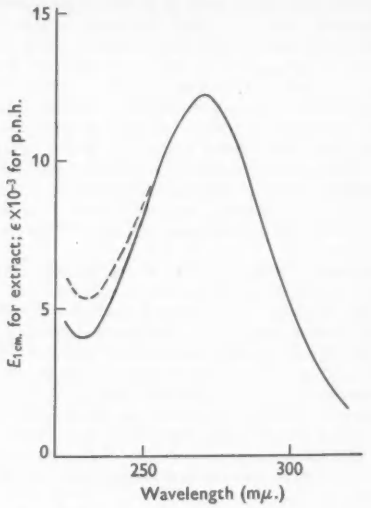

Fig. 1. Ultraviolet absorption spectra in 0·1 N-NaOH of an arbitrary dilution of an extract of locust faeces, – – –, and of *p*-nitrohippuric acid (p.n.h.), ——; $\lambda_{max.}$ 271, $\epsilon_{max.}$ 12200.

p-*Aminobenzoic acid*

Qualitative experiments. Ten mature adult locusts, previously fed on fresh grass, were injected with 0·5 mg. each of sodium *p*-aminobenzoate, kept without food for 48 hr. and the droppings collected over this period. The faeces were made alkaline with a few drops of conc. NH_3 and extracted in the usual way. The ethanolic solution was chromatographed in butanol:water on Whatman no. 4 paper till the front had run about 30 cm. A strip cut from the edge of the sheet after diazotization and coupling showed two coloured zones. A large one, R_F 0·5–0·8, was shown by paper chromatography after elution with water to consist of unchanged *p*-aminobenzoic acid. The slow moving band

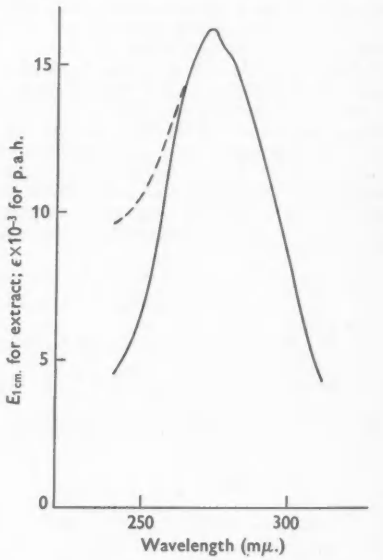

Fig. 2. Ultraviolet absorption spectra in 0·1 N-NaOH of an arbitrary dilution of an extract of locust faeces, – – –, and of *p*-aminohippuric acid (p.a.h.), ——; $\lambda_{max.}$ 273, $\epsilon_{max.}$ 16200.

Table 2. *Formation of* p-*aminohippuric acid* (*PAH*) *from* p-*aminobenzoic acid* (*PAB*) *by individual locusts*

Temp. (°)	PAB injected (μg.)	Period of collection of faeces (hr.)	PAB + PAH in faeces		PAH in faeces	
			μg.	% dose	μg.	% dose
35	310	72	290	94	118	28
			300	97	78	18
			190	61	50	12
			255	82	62	14
			230	74	62	14
	375	24	356	95	48	9
			250	67	32	6
			338	90	66	12
			213	57	74	14
25	310	72	240	78	90	21
			265	86	148	34
			170	55	75	18
			255	82	55	13
			300	97	96	22
			265	86	78	18
	375	24	200	53	24	5
			250	67	50	9
			375	100	24	5

(R_F 0–0·15) was eluted with 0·1 N-NH_3 and further purified by chromatography on paper with butanol:acetic acid:water (4:1:5, v/v). The diazo-reacting zone on this chromatogram was eluted with water and shown to have the same chromatographic properties as *p*-aminohippuric acid in all the solvents used (Table 1).

Its ultraviolet absorption spectrum in 0·1 N-NaOH (Fig. 2), had a band at 273 mμ. In acid no specific absorption was found in this region.

The conjugate was hydrolysed with N-H_2SO_4 at 100° and the presence of *p*-aminobenzoic acid was demonstrated on paper chromatograms. Chromatograms of the hydrolysate run in butanol:acetic acid:water (4:1:5, v/v) showed a spot corresponding with the position of a reference spot of glycine (R_F 0·21) which reacted with ninhydrin and gave the green colour characteristic of glycine when sprayed with the phthalaldehyde reagent. Another portion of the hydrolysate was evaporated to dryness *in vacuo* and converted into the dinitrophenyl derivative which was then chromatographed on buffered paper in water saturated chloroform:butanol (93:7, v/v) (cf. Krol, 1952). The dinitrophenyl derivative from the extracts ran at the same speed as the authentic glycine derivative (R_F 0·26) and was readily distinguished from the ornithine (R_F 0·76) and alanine (R_F 0·65) derivatives.

Quantitative experiments. Some figures for the excretion of *p*-aminohippuric acid are quoted in Table 2. In other experiments it was observed that most of the injected *p*-aminobenzoic acid was eliminated in the first 24 hr. after dosing, though traces of diazotizable amines were detectable in the faeces up to 2 weeks later. After the initial excretion of *p*-aminobenzoic acid the proportion of glycine conjugate in each day's excretion rose. In ten immature adults which each received 800 μg. *p*-aminobenzoic acid, the combined faeces of the second and third days after dosing contained 21–34 μg. (mean 26 μg.) of *p*-aminohippuric acid and only 22–26 μg. (mean 23 μg.) of *p*-aminobenzoic acid. Since some reports suggest that detoxication mechanisms in insects may be affected by temperature (e.g. Sternburg & Kearns, 1952; Butts, Chang, Christensen & Wang, 1953), the conjugation of *p*-aminobenzoic acid was measured at 25 and 35° on locusts from the same hatching. No significant difference at the two temperatures could be observed (Table 2).

Salicylic acid. Twelve immature adult locusts were injected with 0·5 mg. each of sodium salicylate in aqueous solution (0·01 ml.). Faeces collected 24 hr. later were extracted with water and ethanol. This extract was separated on a large-scale paper chromatogram in solvent 1 on Whatman no. 4 paper. In addition to zones corresponding with salicylic acid and salicyluric acid a third unidentified material fluorescing in ultraviolet light with R_F about 0·2 was present. The zone corresponding to salicyluric acid was eluted with water, and the presence of this compound confirmed by comparison with authentic material on paper chromatograms run in the solvents in Table 1. Insufficient material was available for spectroscopic characterization and the degree of conjugation of salicylic acid appeared to be smaller than the other acids studied.

Metabolism of hippuric acids. Three groups, each of six immature adult locusts were injected with 1 mg. each of the sodium salts of hippuric acid, *p*-aminohippuric acid and *p*-nitrohippuric acid. Faeces from each group were collected after 24 hr. and extracted as described above. Paper chromatography of these extracts showed in each case that the glycine conjugate was excreted unhydrolysed. In the case of *p*-nitrohippuric acid a very small trace of *p*-aminohippuric acid was found in the extracts of the faeces.

DISCUSSION

Each of the acids studied was excreted to some extent as a glycine conjugate, and no evidence was found of any other conjugation of the carboxyl group. As we have previously observed with the acetylation mechanism (Myers & Smith, 1953), the capacity of the locust for detoxication by conjugation is low. With *p*-aminobenzoic acid only about one-fifth of the administered material was recovered as the conjugated form, and this conjugation was

not appreciably affected by temperature. Most of the material given was eliminated in the first few days after dosing, but small amounts continued to be excreted for several weeks. Even with the much smaller quantities eliminated on these later days, about half was still unconjugated.

The reduction of the nitro group in *p*-nitrobenzoic acid which was observed indicates the presence of another possible detoxication mechanism in locusts, since reduction of nitro groups often lowers toxicity (cf. Smith *et al.* 1953). This reduction is not unexpected since Dennel (1949) has observed that insect blood has a low redox potential.

The formation of hippuric acids in the locust is interesting in connexion with the comparative biochemistry of nitrogen metabolism. Locusts differ from the common experimental animals in that uric acid is a major end-product of their nitrogen metabolism (Chauvin, 1941). The hen, which also eliminates most of its nitrogen as uric acid, uses ornithine for the detoxication of aromatic acids, and it is sometimes suggested (Quick, 1948; Baldwin, 1948) that other animals with uricotelic metabolism, such as reptiles, may also form ornithuric acids. The only reptile which appears to have been studied is a turtle, which produced hippuric acids (Komori & Sendyu, 1926), but it was not stated whether this turtle had a ureotelic or uricotelic metabolism (cf. Baldwin, 1952). The present work suggests that there may be no general connexion between uric acid production and the use of ornithine as a conjugating agent.

SUMMARY

1. The metabolism of benzoic, *p*-nitrobenzoic, *p*-aminobenzoic and salicylic acids has been studied in the adult locust.

2. The glycine conjugates of these acids have been identified in the excreta by spectroscopic and paper chromatographic techniques.

3. Quantitative measurements showed that about 20% of *p*-aminobenzoic acid was converted into *p*-aminohippuric acid.

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Some Observations on Volatile Fatty Acids in the Sheep's Rumen

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A number of investigations of the volatile fatty acids (VFA's) of the rumen have been reported since the subject was reviewed by Elsden & Phillipson (1948). Particular interest has focused on rumen VFA's other than acetic, propionic and butyric acids. McClymont (1951) made a detailed study of the higher VFA's in the rumen of an ox fed various diets, and Gray, Pilgrim, Rodda & Weller (1952) used a combination of liquid/liquid partition chromatography and chemical methods to demonstrate the presence of formic, *iso*butyric and hexanoic acids, a valeric acid isomer and possibly heptanoic acid in the sheep rumen. The analysis of mixtures of VFA's has been greatly simplified, however, by the development of the method of James & Martin (1952) using gas/liquid partition

chromatography. Shazly (1952*a*) used this method to demonstrate unequivocally that *iso*butyric acid and one or more branched-chain valeric acid isomers occur in the rumen of sheep fed various diets. The production of acetate, propionate, butyrate, valerate and hexanoate by a Gram-negative coccus isolated from the rumen of the sheep has been reported by Elsden & Lewis (1953). Gray *et al.* (1952) have used acids labelled with ^{14}C to show that valeric acid may be synthesized in the rumen from acetic and propionic acids.

The work of Shazly (1952*a*, *b*) relating protein degradation in the sheep rumen to branched-chain VFA production has been extended in the present studies, which were undertaken at the suggestion of Dr R. L. M. Synge. In the first part of the work, which was carried out at the Rowett Research Institute, complete analyses were made of the VFA's produced in the rumen of sheep fed various high-protein diets. Chromatographic evidence was obtained of the presence of *iso*valeric and 2-methylbutyric acids, the two naturally occurring branched-chain valeric acid isomers, but VFA's higher than C_5 were not observed. The investigations were completed at the A.R.C. Institute of Animal Physiology. A partially purified sample of dextrorotatory 2-methylbutyric acid was isolated from the rumen contents of sheep fed a casein-rich diet.

MATERIALS AND METHODS

The experiments at the Rowett Research Institute were carried out using Cheviot ewes fitted with permanent rumen fistulae. In later work, at Babraham, Clun Forest sheep with rumen fistulae were employed.

Volatile fatty acids. Racemic 2-methylbutyric acid and *iso*valeric acid were synthesized by carbonation of the Grignard complexes of *sec.*-butyl and *iso*butyl chlorides respectively, as described by Gilman & Kirby (1932) for 2-methylbutyric acid. Other VFA's used for control purposes were 'laboratory reagent' quality (British Drug Houses Ltd.) and were redistilled before use. Each acid had the calculated equivalent weight on titration with alkali, and was homogeneous when examined by the chromatographic methods mentioned below. The 2-methylbutyric acid was partially resolved by fractional crystallization of the brucine salt (Schutz & Marckwald, 1896); final preparations showed the following specific rotations (measured in 1 dm. tube):

$$[\alpha]_D^{22} = +12{\cdot}9 \pm 0{\cdot}01°: [\alpha]_D^{22} = -6{\cdot}7 \pm 0{\cdot}01°.$$

The reported value of the dextrorotatory form is

$$[\alpha]_D^{20} = +17{\cdot}85°$$

(Marckwald, 1899).

Analysis of volatile fatty acids. The gas/liquid partition-chromatography method of James & Martin (1952) was employed at a temperature of 135° using methyl cellosolve in the heating jacket. Satisfactory separations of the branched-chain valeric acid isomers were obtained by using an 11 ft. column of modified composition. The amount of stearic acid in the liquid phase (DC 550 silicone, Albright and Wilson Ltd., Oldbury, Birmingham) was increased from 10 to 15% (w/w). A nitrogen flow rate of 15 ml./min. was used. All percentages of VFA's are expressed on a molecular basis.

Determination of total VFA's in rumen liquor. The general procedure of McAnally (1944) was followed. Rumen contents were strained through muslin, deproteinized with an equal volume of N-H_2SO_4 saturated with $MgSO_4$, $7H_2O$ and filtered; 2–5 ml. of the filtrate were distilled in a Markham still (Markham, 1942). Three 50 ml. portions of distillate were collected, and titrated with 0·02 N-NaOH under CO_2-free conditions, using phenolphthalein as indicator. When large samples of rumen VFA's were required for fractionation by liquid/gel partition chromatography, the filtrate (50 ml.) was steam distilled and 1–1·5 l. of distillate were collected.

Preliminary fractionation of rumen VFA's. Since only relatively small amounts of C_4 and C_5 VFA's are normally present in sheep rumen liquor, a preliminary concentration of the higher acids was effected by liquid/gel partition chromatography, using the method of Elsden (1946). Samples containing about 1 m-mole of VFA were fractionated on 40 g. silica-gel columns. A fresh column was used for each sample. The bands containing the C_4 and C_5 acids were collected together and titrated with 0·02 N-NaOH under CO_2-free conditions; the salts of the acids were retained for analysis by gas/liquid partition chromatography. Collection of eluate was commenced early in the development of the chromatogram to ensure that any fast-moving bands of higher VFA's present in amounts too small to be observed visually were collected with the C_4 and C_5 acids. The propionic acid band was also collected and titrated, and the acetic acid content of the mixture determined by difference. Formic acid, if present, is not distinguished from acetic acid by this method.

Table 1. *Diets of fistulated Cheviot ewes*

Air-dried materials/24 hr.	g. N/day (approx.)
1. 300 g. groundnut meal, 300 g. ground maize, 300 g. hay	24
2. 100 g. casein, 300 g. ground maize, 300 g. hay	21
3. 750 g. ground maize, 300 g. hay	15
4. 600 g. hay	7

RESULTS

VFA analysis of rumen contents of sheep receiving protein-rich rations

Four ewes were maintained on the diets shown in Table 1. The sheep received half of the daily ration at 12 hr. intervals (8.0 a.m. and 8.0 p.m.) and were trained to eat the food within 1 hr. It was planned to feed the different rations to each sheep in turn, but one of them (sheep 722) was lost after the first part of the experiment. The control diet of hay was therefore eliminated and for the remainder of the experiment only three sheep on the high-protein

Table 2. *Complete analysis of rumen volatile fatty acids (VFA's)*

Date	Sheep	Diet (Table 1)	Time after feeding (hr.)	Total VFA concentration (m-moles/l.)	Molecular percentages of individual VFA's						
					Acetic	Propionic	*iso*Butyric	Butyric	*iso*Valeric	2-Methyl-butyric	Valeric
19. xii. 51	43	Groundnut	2	184	60	26	0·9	10·1	0·9	0·5	1·6
			5	142	44	32	1·9	17·4	1·4	0·7	2·6
			8	97	41	34	2·4	19·0	0·9	0·9	1·8
	716	Maize	2	89	46	32	0·5	18·0	1·3	0·4	1·8
			5	89	56	28	0·4	13·3	1·0	0·3	1·0
			8	104	56	32	0·5	8·9	0·9	0·4	1·3
	718	Casein	2	77	60	22	2·5	7·9	2·8	1·7	3·1
			5	87	70	18	1·3	7·3	1·1	1·0	1·3
			8	92	68	18	1·4	9·0	1·4	1·0	1·2
	722	Hay	2	117	81	13	0·6	4·3	0·3	0·3	0·5
			5	113	77	19	0·5	2·4	0·3	0·3	0·5
			8	120	78	18	0·4	2·9	0·2	0·2	0·3
24. i. 52	43	Maize	2	102	52	35	0·2	12·0	0·3	0·2	0·3
			5	102	50	24	0·5	20·0	0·7	0·2	4·6
			8	94	48	18	0·5	28·9	0·7	0·1	3·8
	716	Casein	2	103	51	25	3·8	10·3	3·6	2·2	4·1
			5	97	58	21	3·1	10·8	2·7	2·4	2·0
			8	95	57	24	2·8	10·3	1·9	1·7	2·3
	718	Groundnut	2	111	43	26	2·2	21·6	2·2	1·9	3·1
			5	122	45	26	2·7	18·8	2·7	1·6	3·2
			8	103	37	28	3·5	24·1	2·4	1·5	3·5
21. ii. 52	43	Casein	2	131	53	24	3·5	13·0	1·9	2·1	2·5
			5	132	48	21	3·2	19·8	2·7	2·4	2·9
			8	113	53	26	2·4	12·0	1·9	1·6	3·1
	716	Groundnut	2	146	48	23	1·9	21·9	1·5	1·1	2·6
			5	143	51	27	2·3	14·4	1·9	1·5	1·9
			8	119	49	29	2·0	16·1	0·9	0·8	2·2
	718	Maize	2	116	—	—	—	—	—	—	—
			5	122	41	30	3·3	19·0	1·8	1·2	3·7
			8	94	—	—	—	—	—	—	—

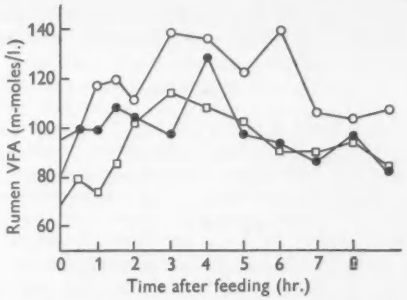

Fig. 1. Changes in rumen VFA concn. (m-moles/l.) after feeding of sheep maintained on diets rich in casein, ●—●; groundnut, ○—○; and flaked maize, □—□.

rations were employed. When the diets were changed the animals were not sampled for at least 1 month, to allow adaptation to the new diets. Rumen samples for complete VFA analysis were taken at 2, 5 and 8 hr. intervals after feeding, and the analytical results are shown in Table 2. As a background to these analyses, smaller samples for determination of total rumen-VFA's were taken at short intervals throughout the same day. The results of a typical experiment are shown in Fig. 1.

Since the procedures described above for the complete analysis of rumen VFA's do not distinguish formic from acetic acid, a series of samples of rumen VFA's from the same sheep were examined by gas/liquid chromatography without preliminary liquid/gel partition. Traces of formic acid were occasionally observed, but in no instance did this exceed 1% of the total VFA's.

Partial purification of the 2-methylbutyric acid of rumen contents

The figures for 2-methylbutyric acid shown in Table 2 are based on the size of the zone observed when the rumen VFA's were examined by gas/liquid chromatography. The chromatographic evidence for the occurrence of 2-methylbutyric acid in rumen contents is summarized below.

Addition of synthetic 2-methylbutyric acid to a sample of rumen VFA's suspected to contain this acid did not increase the number of bands obtained on the gas/liquid chromatogram, but only enlarged the '2-methylbutyric acid' band. In addition, a sample of rumen VFA's was examined on the gas/liquid chromatogram (11 ft. column) and the titration cell contents were changed after the emergence of the *iso*valeric acid band. The next band was collected and recovered from the cell contents by steam distillation. This acid was again examined chromatographically, alone and mixed with synthetic 2-methylbutyric acid. In each case only one band was observed, which emerged after a time which had been established by control experiments to be characteristic for 2-methylbutyric acid under the operating conditions employed.

The separation of 2-methylbutyric acid from the other naturally occurring valeric acid isomers present in rumen contents by techniques other than gas/liquid chromatography has not been reported, although the two branched-chain valeric acids can be separated together as a mixture from valeric acid by the liquid/gel partition method of Moyle, Baldwin & Scarisbrick (1948). Since gas/liquid chromatography has not yet been developed to operate on a preparative scale, further evidence for the occurrence of 2-methylbutyric acid ('active' valeric acid) in rumen contents has been obtained by examining the optical activity of a partially purified sample. The higher VFA's from a large sample were concentrated by successively partitioning the VFA mixture between ether and water at pH 4·5. The 'active' valeric acid content of the resulting acid mixture was compared with the optical activity of the solution.

A sample of rumen contents (700–800 ml.) was taken 4 hr. after feeding from a sheep receiving a casein-rich diet (1 kg. hay (N, 1·3%) and 150 g. casein/day), strained through muslin and treated with an equal vol. of N-H_2SO_4 saturated with $MgSO_4$, $7H_2O$. After centrifuging (2000 *g*, for about 20 min.) the supernatant (1350 ml.) was extracted with ether in a continuous extraction apparatus (Vogel, 1951) until the VFA content of the aqueous layer was reduced to about 5% of its original value. The VFA's in the ether were then removed by shaking with a slight excess of alkali. This procedure was repeated 3 days later with a further 700 ml. of rumen contents obtained from the same sheep. The extracted VFA's were combined, the total VFA was determined (125 m-moles) and a sample was analysed chromatographically. Acetic (60%), propionic (17%), *iso*butyric (4%), butyric (10%), *iso*valeric (approx. 3%), 2-methylbutyric (approx. 2%) and valeric acid (approx. 4%) were present. An aqueous solution of the extracted VFA's was adjusted to pH 4·5 with 50% (w/v) aqueous H_3PO_4, diluted to 250 ml. and shaken with an equal volume of water-saturated ether. The ether layer was then separated, extracted with a slight excess of N-NaOH, and the VFA fraction so obtained was partitioned between ether and water at pH 4·5 as before. The aqueous layers were rejected at each stage and the course of the higher-VFA separation was followed by analysis. This procedure was repeated six times, when 16 m-moles of VFA remained. At this stage, an effort was made to remove any optically active non-acidic substances that may have been present by steam distillation under alkaline conditions. The solution was then acidified and the acids were steam distilled. After neutralization with NaOH, the distillate was concentrated to a small volume, the pH adjusted to 7·0 with 2 N-H_2SO_4 and the solution made up to 20 ml. The VFA content of the solution was determined (14·1 m-moles), and a sample was analysed for VFA's. *Iso*butyric (10%), butyric (13%), *iso*valeric (28%), 2-methylbutyric (13%) and valeric acids (36%) were present. The 2-methylbutyric acid content of the solution was thus 0·93% (w/v). Polarimetric observation of the solution in a 4 dm.

tube showed a rotation of $+0{\cdot}39°$, or $[\alpha]_D^{22} = +10{\cdot}5 \pm 0{\cdot}3°$ (as Na salt but calculated for free acids; c, 0·93) assuming that only 2-methylbutyric acid contributed to the optical activity. A solution of the sodium salt (pH 7·0) of partially resolved dextrorotatory 2-methylbutyric acid

$$([\alpha]_D^{22} = +12{\cdot}9°)$$

was prepared at a concentration of 1·29 %, i.e. equivalent in optical rotatory power to the solution containing isolated 2-methylbutyric acid, assuming that (*a*) the specific rotation of the dextrorotatory acid is $[\alpha]_D^{20} = +17{\cdot}85°$ (Marckwald, 1899), and (*b*) that the optical activity of the isolated VFA solution is due only to dextrorotatory 2-methylbutyric acid. The artificial solution contained in addition the other acids present in the isolated sample at similar concentrations in order to examine their possible effect on the optical activity of the 2-methylbutyric acid. Polarimetric observation of the solution in a 4 dm. tube gave a rotation of $+0{\cdot}38°$, or $[\alpha]_D^{22} + 7{\cdot}4 \pm 0{\cdot}3°$ (same basis as for natural material). The material in rumen liquor, if isolated, would on this basis have $[\alpha]_D^{22} = \frac{10{\cdot}5 \times 12{\cdot}9}{7{\cdot}4} = +18{\cdot}3°$, in good agreement with the value recorded by Marckwald (1899).

Rumen VFA's of fasted sheep

Rumen samples for VFA analysis (without preliminary liquid/gel fractionation) were taken from two sheep receiving diets rich in casein (150 g. casein, 1 kg. hay (N, 1·3 %)/day) and dried grass (600 g. dried grass (N, 2·6 %), 200 g. hay (N, 1·3 %)/day), respectively, over a 48 hr. period after feeding. The total VFA level fell steadily (Fig. 2) but the concentration of the branched-chain VFA's (*iso*butyric, *iso*valeric and 2-methylbutyric acids) showed little change. Formic acid was absent from all samples, but traces of C_6 acids, tentatively identified as γ-methyvaleric and hexanoic acids by the times at which they emerged from the gas/liquid partition column, were observed in the later samples.

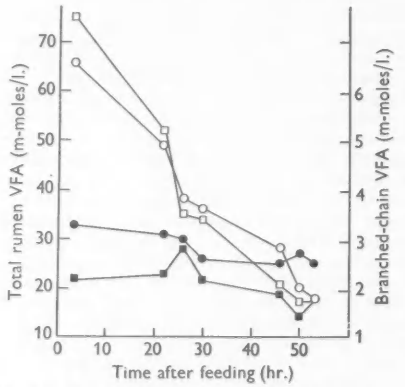

Fig. 2. Changes in total and branched-chain VFA concn. (m-moles/l.) in the rumen of fasted sheep maintained on diets rich in casein (total VFA's, ○—○, branched-chain VFA's, ●—●) and dried grass (total VFA's, □—□, branched-chain VFA's, ■—■).

DISCUSSION

The occurrence of dextrorotatory 2-methylbutyric acid in the sheep rumen has been established. Measurable quantities of *iso*butyric acid and the three naturally occurring valeric acid isomers were observed in each sample of rumen contents examined (Table 2). Some correlation between the quantity of protein fed and the amount of branched-chain VFA's produced can be seen; this aspect of protein degradation in the rumen has been examined more fully by Shazly (1952*a*). The comparatively large amounts of valeric acid observed, particularly on a maize diet, support the findings of Gray *et al.* (1952) on the production of valeric acid in the rumen. The absence of C_6 and C_7 VFA's in rumen samples taken from sheep under normal conditions was unexpected in view of the findings of McClymont (1951), who observed a C_6 acid in the ox rumen, and Gray *et al.* (1952), who reported the occurrence of C_6 and C_7 acids in the sheep rumen. Traces of C_6 acids were found in the rumen of fasted sheep. Small amounts of formic acid (1 % of the total VFA's) were infrequently observed in rumen samples, in contrast to the observations of Gray *et al.* (1952) who reported 0–5 % formate in the rumen VFA's of sheep fed wheaten hay or lucerne hay.

The amounts of branched-chain VFA's in the rumen almost certainly depends on the rate and extent of degradation of ingested and microbial protein and on the rate of absorption of the acids produced. If the latter factor is fairly constant, the relatively slight changes in rumen branched-chain VFA level observed on fasting probably indicates increased microbial degradation which compensates for the steadily decreasing amounts of ingested protein present in the rumen.

The production of ammonia in the rumen as a result of protein degradation was demonstrated by McDonald (1948, 1952). Shazly (1952*a*) measured ammonia and VFA production in the rumen of sheep fed various diets, and ruminal ammonia levels in sheep maintained on the diets shown in Table 1 have been reported (Annison, Chalmers, Marshall & Synge, 1954). The nutritional significance of the extent to which ingested protein is degraded in the rumen has been discussed by Chalmers, Cuthbertson & Synge (1954), Chalmers & Synge (1954) and Annison *et al.* (1954).

Recent investigations have indicated that branched-chain fatty acids are probably more widely distributed in animal tissues than was once considered likely (cf. Shazly, 1952*a*). Branched-chain acids have been revealed as minor constituents

of wool grease (Weitkamp, 1945), butterfat (Hansen & Shorland, 1951, 1952; Hansen, Shorland & Cooke, 1951), ox fat (Hansen, Shorland & Cooke, 1952*a*) and mutton fat (Hansen, Shorland & Cooke, 1952*b*, 1953), and substantial quantities have been found in the body fat of whales and porpoises (Lovern, 1934). Optically active 4-methylhexanoic acid (contaminated with traces of 2-methylhexanoic acid) has been isolated from the coccygeal glands of ducks, together with appreciable amounts of other unidentified alkyl-substituted acids (Weitzel & Lennert, 1951). Brouwer & Nijkamp (1953) have reported the occurrence of 2-methylbutyric and *iso*valeric acids in the hair grease of dogs.

The stereochemistry of the C_{15}, C_{17} and C_{19} *ante-iso* acids isolated from mutton fat (Hansen *et al.* 1952*b*, 1953) and wool fat (Weitkamp, 1945) has been discussed by Klyne (1953), who showed that these acids are almost certainly L-methyl alkanoic acids. Dextrorotatory 2-methylbutyric acid is also a (+)*ante-iso* acid or L-methyl alkanoic acid (Klyne, 1953) and would arise by degradation of L-isoleucine. This supports the postulated origin of these VFA's from protein in the rumen and lends support to the suggestion of Shazly (1952*b*) that the branched-chain VFA's present in the rumen might possibly be precursors of the higher branched-chain acids found in animal fats by Hansen and his co-workers.

SUMMARY

1. The presence of *iso*butyric, *iso*valeric and 2-methylbutyric acids in the rumen of sheep maintained on various diets has been demonstrated.

2. Polarimetric observation of a partially purified sample of 2-methylbutyric acid isolated from the rumen contents of a sheep fed a casein-rich diet revealed that only the dextrorotatory form was present.

3. Volatile fatty acids (VFA's) higher than C_5 have not been observed in the sheep's rumen under normal conditions, although traces of C_6 acid have been found in the rumen of fasted sheep. Traces of formic acid were occasionally observed.

4. The changes in the VFA content of the rumen of sheep when fasted have been examined. Although the total VFA level fell steadily, the concentration of the branched-chain VFA's showed only a slight decrease.

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Studies on the Metabolism of Semen

9. EFFECT OF SURFACE ACTIVE AGENTS WITH SPECIAL REFERENCE TO THE OXIDATION OF SUCCINATE BY SPERMATOZOA

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In the course of investigations on the respiration and the cytochrome–succinic oxidase system of ram spermatozoa (Mann, 1945, 1951*a*, *b*; Humphrey & Mann, 1949) two phenomena have been observed: (i) it was found that as the result of freezing and subsequent thawing, or after prolonged storage of semen *in vitro*, there was a leakage of cytochrome *c* from the spermatozoa into the surrounding fluid; (ii) it was noticed that, whereas the oxygen consumption of suspensions of fresh, washed spermatozoa was but slightly increased by the addition of succinate, sperm cells which have become senescent or have undergone some damage with resulting lowered motility and respiration, responded to the addition of succinate by a markedly increased uptake of oxygen.

We have now found that it is possible to produce the stimulating effect of succinate on the rate of sperm respiration, characteristic of senescent or damaged spermatozoa, by exposing the sperm cells to the action of certain ionic and non-ionic detergents. Under the influence of the surface-active agents, motility, fructolysis and respiration were suppressed, but it was possible to raise the rate of oxygen consumption of the detergent-treated sperm by the addition of sodium succinate.

EXPERIMENTAL

Spermatozoa. The material used in the present investigation consisted of ram semen collected by means of an artificial vagina (Walton, 1946).

Oxygen uptake and fructolysis. In whole semen, respiration and fructolysis were determined at 37°, after a tenfold dilution of the semen with 'Ringer-phosphate-fructose' which had the following composition: 100 ml. 0·9% (w/v) NaCl + 4 ml. 1·15% (w/v) KCl + 1 ml. 2·11% (w/v) KH_2PO_4 + 1 ml. 3·82% (w/v) $MgSO_4$, $7H_2O$ + 21 ml. 0·05% fructose solution in 0·25 M phosphate buffer, pH 7·4 (20 ml. 3·55% Na_2HPO_4 + 1 ml. N-HCl). From the semen thus diluted, 2 ml. were used for the manometric determination of oxygen uptake in a Barcroft differential manometer, and 6 ml. were placed in an incubation tube (0·8 cm. in diameter), from which 1 ml. samples were withdrawn at 30 min. intervals, deproteinized, and used for fructose determination (Mann, 1946, 1948*a*, *b*). Deproteinization was carried out in centrifuge tubes by diluting each 1 ml. sample with 3 ml. water and adding 0·5 ml. 5% (w/v) $ZnSO_4$, $7H_2O$ and 0·5 ml. 0·3 N-$Ba(OH)_2$; in the centrifuged extract the colour reaction was developed according to Roe (1934): to 2 ml. of the extract were added 2 ml. 0·1% ethanolic solution of resorcinol and 6 ml. 10 N-HCl, the mixture was heated 10 min. at 85° and, after cooling, the colorimetric determination was carried out in the Hilger Spekker Absorptiometer (1 cm. cell; Spectrum Blue-green Ilford filter no. 603, transmission 470–520 mμ.).

In some experiments, washed sperm suspensions were used instead of whole semen. For this purpose, semen diluted ten times with Ringer-phosphate-fructose, was centrifuged for 10 min. at 1200 *g* and supernatant fluid was removed; the sperm were resuspended by gentle mixing with a small amount of Ringer-phosphate or Ringer-phosphate-fructose, and brought up to the volume of ten-times-diluted semen.

Effect of succinic acid. The effect of succinic acid on the respiration of spermatozoa was followed manometrically, in the presence of 6 mg. sodium succinate. When cytochrome *c* has been used, the amount added was 0·2 ml. of a 1% solution; cytochrome *c* was prepared from heart muscle by the method of Keilin & Hartree (1945). For manometric experiments, spermatozoa were used either as semen suitably diluted with Ringer-phosphate-fructose or Ringer-phosphate, or as a suspension of washed cells.

Detection of cytochrome c. The leakage of cytochrome *c* from the spermatozoa into the surrounding medium was observed spectroscopically, in the Zeiss Microspectroscope, by a method outlined in an earlier paper (Mann, 1951*a*).

Surface-active agents. The following substances were used. Hexadecyltrimethylammonium bromide, generally called cetyltrimethylammonium bromide (CTAB), mol.wt. 364; 2-phenoxyethanol (phenoxetol), mol.wt. 138; 'Lubrol W' (a cetyl alcohol/ethylene oxide condensation product manufactured by Imperial Chemical Industries Ltd.), sodium dodecyl sulphate, mol.wt. 288; dodecyl amine hydrochloride, mol.wt. 222; saponin; 'Teepol' (a wetting agent manufactured by the Shell Co. Ltd.), and sodium taurocholate, mol.wt. 538.

RESULTS

Spermicidal effect of surface-active agents

Of all the substances which have been tested, cetyltrimethylammonium bromide (CTAB), a cationic detergent, had the strongest influence on fructo-

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lysis and motility. The response of sperm to CTAB varied somewhat from one sample of ram semen to another; 0·0003–0·0006 M CTAB was enough to reduce the rate of fructolysis by 50 % and concentrations from 0·0008 to 0·0012 M suppressed completely both motility and fructolysis. Sodium dodecyl sulphate used in 0·0005–0·001 M concentration produced a 50 % and in 0·0015 M, a complete inhibition of fructolysis; motility examined 30 min. after the addition of 0·0015 M dodecyl sulphate to semen diluted with Ringer-phosphate-fructose was nil. Under similar conditions, 2-phenoxyethanol (phenoxetol) acted in a concentration of 0·02–0·03 M. The effects of these three substances on sperm fructolysis are illustrated in Fig. 1.

The oxygen uptake of dilute semen was also affected by these substances but the full effect on respiration usually became obvious somewhat later than on fructolysis.

Of practical interest is the inhibition of sperm motility, fructolysis and respiration by 'Teepol', in view of the common use of this wetting agent for cleaning of glassware. In a dilution 1:750, 'Teepol' produced a 25 % inhibition of both fructolysis and respiration; at 1:300 it completely abolished sperm metabolism and motility.

The least active of all substances tested was saponin, of which 0·1 % was required to achieve complete inhibition.

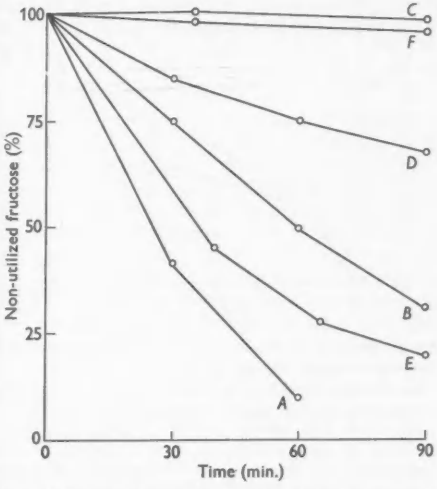

Fig. 1. Effect of hexadecyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS) and 2-phenoxyethanol (phenoxetol) on sperm fructolysis; ram semen diluted with 9 vol. Ringer-phosphate-fructose and incubated at 37°; *A*, no additions; *B*, 0·0005 M CTAB; *C*, 0·001 M CTAB; *D*, 0·001 M SDS; *E*, 0·01 M phenoxetol; *F*, 0·03 M phenoxetol.

Several attempts were made to reverse the inhibition caused by CTAB, dodecyl sulphate and phenoxetol, by washing the detergent-treated sperm with fresh Ringer-phosphate-fructose. However, the inhibition proved to be irreversible in all instances so far examined.

Leakage of cytochrome c

The spermicidal action of all the surface-active agents used in this study, was accompanied by a leakage of cytochrome *c* from the spermatozoa into the external medium. The amount of extracellular cytochrome appeared to be roughly proportional to the concentration of the added agent and increased on incubation. In every case where motility was brought to a standstill by a surface-active agent, the band of cytochrome *c* at 550 mμ. could readily be detected after centrifugation in the sperm-free supernatant fluid, either directly, or better still, after reduction with sodium dithionite.

Effect of sodium succinate on sperm respiration

Fresh semen or once-washed spermatozoa responded to the addition of succinate by a slightly increased rate of oxygen uptake. The response was more pronounced if succinate was used together with cytochrome *c*; it is not improbable, however, that the observed increase was actually due to the moribund cells rather than the intact spermatozoa. On the other hand, a high percentage increase in oxygen consumption was observed when succinate, either alone or with cytochrome *c*, was added to sperm suspensions which had lost their original respiratory activity as a result of treatment with surface-active agents. In order to demonstrate this effect clearly it is essential to add the correct amount of detergent and to avoid an excess. This point is best illustrated by experiments with CTAB. This compound, when present in excessive concentrations, i.e. above 0·002 M, led not only to a loss of motility and respiratory as well as fructolytic power, but abolished also the ability of sperm to respond to added succinate; this is not unexpected as it is known that CTAB acts in high concentrations as an inhibitor of the succinic oxidase system (Hockenhull, 1948). When, however, CTAB was present in smaller concentrations, the spermatozoa were deprived of motility as well as of fructolytic and respiratory ability, but became at the same time capable of responding to added succinate with an increased oxygen uptake.

The most convincing results were obtained when semen was first incubated with CTAB alone, then the spermatozoa were separated by centrifugation, suspended in fresh Ringer-phosphate, and finally brought into contact with succinate. The procedure in these experiments was as follows.

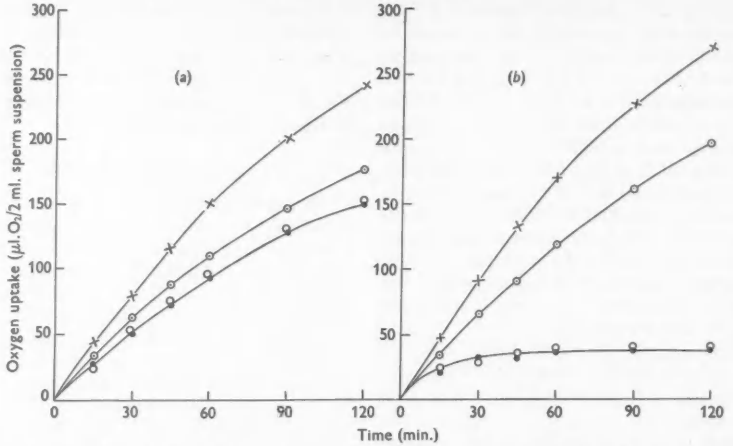

Fig. 2. Effect of succinate on the respiration of spermatozoa obtained by centrifugation of (*a*) semen incubated with Ringer-phosphate, and (*b*) semen incubated with Ringer-phosphate containing 0·002 M CTAB; 2 ml. sperm suspension corresponds to 0·2 ml. semen; for further experimental details see text; ●—●, no addition; ○—○, cytochrome *c*; ⊙—⊙, succinate; ×—×, succinate + cytochrome *c*.

Whole semen (2 ml.) was used; 1 ml. was diluted with an equal volume of Ringer-phosphate, and 1 ml. with an equal volume of a 0·002 M solution of CTAB in Ringer-phosphate. Both samples were incubated in centrifuge tubes for 20 min. at 37° and then centrifuged for 10 min. at 2500 *g*. The supernatant fluids were used for spectroscopic examination, after addition of sodium dithionite. The two centrifuged sperm samples were suspended in Ringer-phosphate and diluted to 10 ml. From each suspension, four 2 ml. samples were pipetted into manometer vessels and the oxygen uptake was recorded (i) in the absence of any additions, (ii) in the presence of succinate, (iii) in the presence of cytochrome *c*, and (iv) with succinate and cytochrome *c* added together.

The spectroscopic examination of the supernatant fluid obtained by centrifugation of semen untreated by CTAB, showed a barely detectable band of cytochrome *c*; this slight leakage of cytochrome being due to high-speed centrifugation itself. The supernatant from the CTAB-treated semen on the other hand, was distinctly pink in colour and showed a very strong band of cytochrome *c*. The measurements of respiratory activity in the untreated and CTAB-treated sperm are recorded in Fig. 2 from which it can be seen that CTAB in the concentration used reduced endogenous respiration and that succinate, particularly if added together with cytochrome *c*, brought about a higher percentage increase of the oxygen consumption in detergent-treated than in the untreated spermatozoa. Although 'respiring' vigorously in the presence of succinate and cytochrome, the detergent-treated spermatozoa were nevertheless completely immotile and devoid of fructolytic activity.

The oxidation of succinate by the detergent-treated spermatozoa was counteracted effectively by malonate (Fig. 3), a fact which is in agreement with the property of malonate to act as a competitive inhibitor of succinic dehydrogenase.

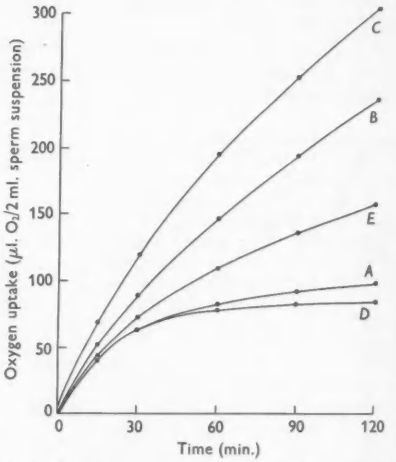

Fig. 3. Effect of malonate on the respiratory activity of detergent-treated spermatozoa; ram semen incubated in the presence of 0·001 M CTAB, 20 min., 37°; spermatozoa separated by centrifugation and suspended in Ringer-phosphate; 2 ml. sperm suspension correspond to 0·2 ml. semen; *A*, no additions; *B*, with succinate; *C*, with succinate and cytochrome *c*; *D*, with 0·01 M sodium malonate, either with or without succinate; *E*, with 0·01 M sodium malonate plus succinate plus cytochrome *c*.

Experiments analogous to those with CTAB, have also been carried out with other substances. In every case where incubation with a given substance rendered the sperm either completely or partly immotile, the reduced rate of oxygen uptake could be increased by the addition of succinate, either alone or with cytochrome *c*. In order to demonstrate clearly the effect of succinate, the following concentrations were used: 0·001 M sodium dodecyl sulphate, 0·002 M dodecylamine hydrochloride, 0·005–0·01 M sodium taurocholate, 0·01 M phenoxetol, 0·1 % 'Lubrol' or saponin.

A series of experiments was also carried out to examine the possibility that substrates other than succinate might be able to increase the respiration of detergent-treated sperm cells. Two groups of substrates were examined: one comprised various substances known to stimulate the normal respiration of intact spermatozoa, such as fructose, glucose, lactate, pyruvate and acetate; the other was represented by substances which ordinarily are unable to bring about a stimulation of respiration, e.g. ethanol, glycerol and citrate. However, such effects as were obtained, were either small or negligible.

Finally, some experiments were performed to find whether the succinic oxidase activity of detergent-treated spermatozoa was confined to the cells or whether it passed partly also into the extracellular medium together with cytochrome *c*. So far, all attempts to demonstrate succinic oxidase activity in the sperm-surrounding fluid have proved negative.

Structural changes in spermatozoa

Detergent-treated and untreated spermatozoa were examined by Dr J. R. G. Bradfield in the electron microscope, with and without metal shadowing. In contrast to the striking biochemical changes, the structural abnormalities observed after incubation of semen with 0·001 M CTAB were exceedingly small. However, incubation with 0·0025 M CTAB caused marked disruption of sperm-head membranes in about 50 % of spermatozoa and fraying of the tail-ends in about 75 %; after treatment with 0·0125 M CTAB, about 25 % of the middle-pieces became distinctly more 'fuzzy' and cast much shorter shadows than did middle-pieces of untreated spermatozoa. Sections of spermatozoa were not prepared.

DISCUSSION

The literature on the question of whether or not succinate supports the respiration of mammalian spermatozoa is full of conflicting statements (for review see MacLeod, 1943; Mann, 1949). Our own experience, based mainly on observations with ram sperm, has been that succinate, unlike fructose and the products of fructolysis, is incapable of supporting the respiration of normal intact spermatozoa, but can increase considerably the oxygen uptake of senescent and already partly immotile spermatozoa, without, however, improving their motility. The present investigation demonstrates that certain surface-active substances (i) are spermicidal, in the sense that they inhibit motility, fructolysis and respiration of spermatozoa, (ii) induce a leakage of cytochrome *c*, and (iii) enable the cells, if used in suitable concentrations, to respond to added succinate by an increased oxygen uptake. All these effects are presumably brought about by some change in the permeability of the sperm cell.

There is considerable evidence to show that under physiological conditions, the surface of the spermatozoon is protected by a lipid-containing outer layer, the so-called lipid capsule or 'manteau lipidique', and it is probable that the surface-active substances act directly on the lipoprotein and lipid constituents of that capsule. Thus, the mechanism of the spermicidal activity of detergents on the sperm may be not altogether different from that underlying the haemolytic action of certain surface-active compounds on erythrocytes and the bactericidal effects of such agents on various micro-organisms (cf. Schwartz & Perry, 1949; Salton, 1951; Hugo & Street, 1952; Pethica & Schulman, 1953). Our findings concerning the behaviour of spermatozoa are of special interest in view of the exceptional resistance of these cells to plasmolytic agents in general. Moreover, our results obtained with detergent-treated spermatozoa to which succinate has been added, indicate that an increase in the oxygen consumption of cells *in vitro* need not coincide with the maintenance of their 'vital capacity'. As a matter of fact, we have shown that an increased oxygen uptake of detergent-treated spermatozoa in response to added succinate, was the direct outcome of far-reaching cellular disorganization. The precise mechanism of the phenomena outlined above requires further study, especially as regards the catalytic activity of the sperm cytochrome–succinic oxidase system.

SUMMARY

1. Certain surface-active compounds, including ionic and non-ionic detergents, were found to inhibit motility, fructolysis and respiration of spermatozoa. Among the substances examined, hexadecyl (cetyl)-trimethylammonium bromide and dodecyl sulphate were particularly effective. Their spermicidal power was assessed quantitatively by measuring the inhibitory influence on fructolysis and respiration.

2. Hexadecyl(cetyl)trimethylammonium bromide, 0·0003–0·0006 M, inhibited the fructolysis of

ram spermatozoa by 50%; 0·0008–0·0012M was required for complete inhibition of fructolysis and motility. Among similarly active substances were dodecyl sulphate, 2-phenoxyethanol, dodecylamine hydrochloride, sodium taurocholate, 'Lubrol' and 'Teepol'.

3. The surface-active compounds induced a change in the permeability of spermatozoa as indicated by the diffusion of cytochrome *c* from the cells into the surrounding fluid.

4. When succinate was added to detergent-treated immotile spermatozoa, the percentage increase of the oxygen uptake was much greater than in intact motile sperm; thus, the stimulating effect of succinate on the respiration of spermatozoa was an indication of a moribund cell population.

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Two Methods for the Determination of Glycogen in Liver

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Several methods for the determination of glycogen in tissues have been described (Good, Kramer & Somogyi, 1933; Morris, 1948; Boettiger, 1946; Seifter, Dayton, Novic & Muntwyler, 1950; van Wagtendonk, Simonsen & Hackett, 1946; van der Kleij, 1951). As we needed a simple technique for the study of the glycogenetic properties of adrenal steroids a survey was made of several techniques often used (see Table 1). For serial determinations the methods of Seifter *et al.* and van der Kleij are attractive, in part because the time-consuming separation of the glycogen by precipitation with alcohol is avoided. However, in Seifter's method this separation can only be omitted if the glycogen content of the liver exceeds 1%, while van der Kleij's technique is not specific for glycogen, since substances like glucose and glucose phosphates, which are known to occur in liver, are also estimated.

The results submitted in this paper show that the process of extracting the glycogen with trichloroacetic acid as proposed by van der Kleij can be used in combination with colorimetric techniques for the estimation of glycogen as described by van Wagtendonk *et al.* (with iodine) and Morris (with anthrone). In this way two methods were developed, which can be used for the serial determination of glycogen in liver. In particular the application of an iodine reagent proved to be very convenient for routine determinations of glycogen. In our hands, the use of this method instead of the more elaborate techniques of Pflüger (1905) and others led to a considerable saving of time and materials. Satisfactory results have been obtained, despite some limitations of the method, which will be discussed in detail.

REAGENTS AND METHODS

Extraction of glycogen from liver with trichloroacetic acid

If the iodine reagent is used for the determination of glycogen in the extracts the concentration of trichloroacetic acid (TCA) should not exceed the limits 4·9–5·1% (w/v). This is checked by titration with 0·1N alkali, and the solution adjusted if necessary by adding water or a concentrated solution of TCA.

Procedure. A 200 mg. sample of liver is weighed on a torsion balance and finely ground with 20 ml. of 5% TCA

in a mortar or preferably in a homogenizer (Potter & Elvehjem, 1936). The precipitate of proteins is filtered off and the clear filtrate submitted to analysis.

Estimation of glycogen in the extracts

(*a*) *With iodine*

Iodine reagent: 16·5 ml. of Lugol's solution, prepared by dissolving 1 g. of iodine and 2 g. of KI in 20 ml. of water, is added to 990 ml. of an aqueous solution, containing 25% (w/v) of KCl.

Procedure. In a colorimeter tube (1·2 cm. diameter) 2 ml. of a liver extract is added to 3 ml. of iodine reagent. After mixing, the optical density is read in a photometer at 650 mμ. against a blank, obtained by adding 2 ml. of 5% TCA to 3 ml. of reagent in the same way. The amount of glycogen is read from a calibration curve, which must be prepared using glycogen of the same origin as the sample to be estimated.

(*b*) *With anthrone*

Anthrone reagent: 0·5 g. of anthrone is dissolved in 250 ml. of 95% (w/v) H_2SO_4. As this reagent deteriorates rapidly it should be freshly prepared before each series of determinations.

Procedure. 2 ml. of the liver extract are pipetted into a test tube calibrated at 20 ml., 2 ml. of 10N-KOH are added and the tube placed in boiling water for 1 hr. After cooling, 1 ml. of glacial acetic acid is added to neutralize the excess of alkali, and the fluid brought up to the mark with water: 2 ml. of the solution are added slowly to 4 ml. of anthrone reagent in a test tube, which is placed in cold water to prevent excessive heating. After mixing by lateral shaking, the tube is placed in boiling water for exactly 10 min. for colour development and cooled with tap water. The optical density is read within 2 hr. in a photometer at 650 mμ. against a blank, which is prepared by submitting 2 ml. of 5% TCA to the same procedure. For the calculation of the amount of glycogen, glucose standards are prepared in each series as follows: 2 ml. of TCA are heated to 100° with 2 ml. of 10N-KOH and the solution is neutralized with 1 ml. of glacial acetic acid as described above. Then 4 ml. of a solution containing glucose (0·15 and 0·60 mg.) are added. After dilution to 20 ml. with water the procedure is continued in the usual way.

Preparation of glycogen

The livers (or muscles) of 10–20 rats were quickly removed and transferred as rapidly as possible to 300 ml. of 30% (w/v) NaOH at 100°. Heating was continued for 2 hr. and the crude glycogen precipitated by adding twice the volume of 96% (v/v) ethanol. After separation, the precipitate was dissolved in a small volume of water and the solution adjusted to pH 3 with dilute HCl. The glycogen was re-precipitated by the addition of an equal volume of ethanol. This process of precipitation was repeated once more, and the resulting substance washed with ethanol and ether and finally dried *in vacuo*. The purity of the glycogen determined after conversion into glucose usually amounted to 90–95%. For these estimations glucose was determined with the Hagedorn & Jensen method. In all experiments optical densities were measured in a Lumetron photometer (model 402 E) provided with monochromatic filters.

Table 1. *Survey of some methods for the determination of glycogen*

	Pflüger (1905)	Good *et al.* (1933)	Morris (1948)	Boettiger (1946)	Seifter *et al.* (1950)	van Wagtendonk *et al.* (1946)	van der Kleij (1951)
Separation of glycogen from the tissues	Destruction by alkali, 2 hr.; precipitation by ethanol	Destruction by alkali, 20 min.; precipitation by ethanol	Destruction by alkali, 20 min.; precipitation by ethanol	Destruction by alkali, 2 hr.; precipitation by ethanol	Destruction by alkali, 20 min.	Destruction by alkali, 2 hr.; precipitation by ethanol	Grinding with trichloroacetic acid
Purification of glycogen	Washings with 66, 85 and 96% ethanol and ether	None	None	Washings with 66, 85 and 96% ethanol and ether	None	None	None
State at estimation	Glucose	Glucose	Glucose	Glucose	Glucose	Glycogen	Glucose
Conversion into glucose	By hydrolysis (3 hr.)	By hydrolysis (2 hr.)	During colour development	During colour development	During colour development	None	During colour development
Final titration	Any glucose determination	Folin & Wu's colorimetric method	Colorimetric method with anthrone	Colorimetric method with diphenylamine	Colorimetric method with anthrone	Colorimetric method with iodine	Colorimetric method with sulphuric acid

RESULTS

Estimation with iodine reagent

Fig. 1 shows the relationship between the optical density of a glycogen-iodine complex and the glucose equivalent of glycogen at various wavelengths. This diagram was constructed with a glycogen sample obtained from rat liver. As these relationships can be represented by straight lines the optical density can be read at different wavelengths. In practice we adopted 650 mμ. as the most suitable one for estimation of glycogen over a wide range of concentrations without necessitating dilution of glycogen-rich liver extracts. The colour intensity of the glycogen-iodine complex decreases with increase in the concentration of TCA in the extract, but within the limits of 4·9–5·1 % TCA, variations in optical density due to different acidity can be neglected.

Glycogen is very stable in the extracts. The differences between estimations of thirty-seven samples from various livers, performed immediately after extraction of the tissues and 18 hr. later, respectively, averaged $+0{\cdot}01\,\% \pm 0{\cdot}03$, the greatest deviation being 0·08 %. The extracts can therefore be left overnight, which adds to the usefulness of the TCA method.

The reproducibility of the technique was investigated by performing duplicate determinations on the same liver. In view of the irregular distribution of glycogen in liver found by Nijs, Aubert & de Duve (1949), adjacent samples of tissue were taken for analysis. In eighteen experiments a mean difference of $0{\cdot}06 \pm 0{\cdot}068$ in percentage liver glycogen was found between duplicate samples. Similar liver samples were analysed using the more elaborate Pflüger method; in eleven experiments a mean difference of $0{\cdot}33 \pm 0{\cdot}225$ was found, so with the iodine method more reproducible results are obtained than with Pflüger's technique.

The calibration curves relating optical density to glucose equivalent have a different slope with glycogen samples of different origin (Fig. 2); this emphasizes the necessity for a standard curve, obtained with glycogen from the same source as the glycogen samples which are estimated. The magnitude of this slope may be used to characterize glycogen for comparative purposes and is termed here the 'colour intensity', being related to the extinction coefficient. Using the data of Fig. 2, values of colour intensity for samples of glycogen from mouse liver, rat liver and rat muscle were calculated to be 1·51, 1·00 and 0·81, respectively. In subsequent experiments the colour intensity of a glycogen was determined by submitting a solution to two determinations, one by iodine-staining and the other by anthrone.

It was found that differences in colour intensity exist between samples of glycogen prepared from the livers of different individuals of one species (rats). The colour intensities were estimated at various wavelengths for five samples of glycogen prepared from different livers (Table 2). Determinations in triplicate were performed on one

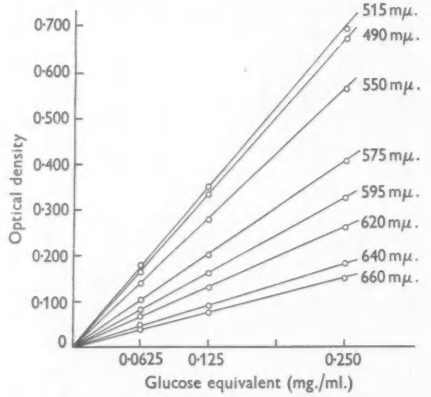

Fig. 1. Relations between optical density of a glycogen-iodine complex and glucose equivalent of glycogen at various wavelengths. A homogenous sample of glycogen from the livers of rats was used. The glucose equivalents indicated refer to 1 ml. of iodine-stained solution.

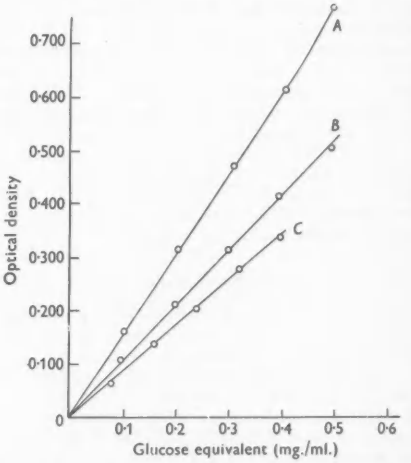

Fig. 2. Calibration curves for glycogen from different sources. Optical densities were read at 650 mμ. The glucose equivalents indicated on the abscissa refer to 1 ml. of iodine-stained solution and were calculated from the estimated purities of the glycogen preparations. *A*, mouse liver; regression-coefficient of the line, 1·51. *B*, rat liver; regression coefficient, 1·00. *C*, rat muscle; regression coefficient, 0·81.

homogeneous sample of glycogen as well. The variations in the values for glycogen from different livers (standard deviations in upper part of the table) exceed significantly those solely due to technical errors (standard deviations in lower part of the table). This was proved statistically by computing a mean square for each part of the table by pooling the standard deviations according to the formula $(\Sigma s^2)/n$. The ratio of these mean squares was found to be 4·36 and indicates a significant difference with $P<0·001$. Hence, colour intensities of glycogen differ from one liver to another.

As the differences between the samples of glycogen existed at all wavelengths, only colour intensities at 650 mμ. were compared in the following experiment. After preliminary starvation, rats were dosed with glucose and the colour intensities of liver glycogen compared with those of normally fed rats (Table 3). The results suggest that following a treatment which effects a rapid deposition of glycogen in the liver the colour intensity of the glycogen increases, but independently of the actual amount of glycogen in the liver. This suggests that newly formed glycogen has the greater colour intensity.

Estimation with anthrone reagent

The estimation of glycogen in liver extracts by means of the anthrone reagent includes a simultaneous hydrolysis of glycogen to glucose, so this estimation is not influenced by the origin of the glycogen. Treatment of liver extracts with hot alkali resulted in an elimination of interfering glucose, without affecting the glycogen (Table 4); this enhances the specifiaty of the method.

A liver incubated at 37° in Krebs-Ringer phosphate buffer is a suitable object to demonstrate the estimation of glycogen in the presence of increased amounts of physiological degradation products, such as can occur in the system to be studied. Experiments were performed with eight livers, incubated at 37° for 15 min. The livers were extracted by grinding 500 mg. of tissue with 30 ml. of TCA. In order to hydrolyse the glycogen, 10 ml. of extract were heated with 0·5 ml. of conc. HCl for 3 hr. at 100°. After neutralization of the acid with alkali and readjusting the quantity of fluid to 10 ml. with water, 10 ml. of 10% TCA were added, giving a final concentration of 5%, as the TCA originally present was decomposed by boiling with hydrochloric acid. A comparable solution was obtained by mixing the components in a sequence which prevented the hydrolysis of the glycogen. In the solutions, glycogen was estimated both including and excluding a treatment with alkali

Table 2. *Colour intensities at various wavelengths for glycogen from the livers of individual rats*

	Wavelength (mμ.)								
	465	490	515	550	575	595	620	640	660
	Colour intensities								
Mean ± S.D. of glycogen preparations from the livers of five different rats	2·12 ±0·23	2·53 ±0·32	2·46 ±0·24	2·24 ±0·24	1·61 ±0·21	1·32 ±0·25	1·06 ±0·15	0·74 ±0·11	0·68 ±0·09
Mean ± S.D. triplicate determinations on one glycogen preparation	2·63 ±0·29	2·78 ±0·07	2·85 ±0·06	2·30 ±0·02	1·64 ±0·05	1·30 ±0·01	1·07 ±0·01	0·73 ±0·02	0·63 ±0·02

Table 3. *Colour intensities at 650 mμ. for glycogen from the livers of individual rats*

Normally fed rats		Glucose-treated rats*	
Liver glycogen (% of fresh wt.)	Colour intensity	Liver glycogen (% of fresh wt.)	Colour intensity
1·76	0·59	3·50	1·38
2·42	0·89	1·36	1·52
5·76	0·95	1·25	1·80
1·34	0·80	1·68	1·85
5·05	0·87		
Av. 3·27	0·82	1·95	1·64

* After fasting for one night each animal received 7 hourly doses of 0·5 g. of glucose subcutaneously. The mean difference between the colour intensities is statistically significant ($t_7=5·21$, $P<0·01$).

Table 4. *Influence of treatment with alkali on glucose and glycogen estimation with anthrone*

		Time of treatment with alkali at 100° (min.)					
		0	15	30	45	60	90
	No. of observations	Recovery of glucose or glycogen (%)					
Glucose (0·60 mg./ml.)	4	100	4	3	4	3	3
Glycogen (glucose equivalent 0·32 mg./ml.)	2	100	104	104	103	99	101

The experiments were performed as indicated in the Methods section.

for 1 hr. at 100° (Table 5). By omitting the treatment with alkali both glycogen and glucose are estimated. After previous hydrolysis of the glycogen the same results are obtained. Hydrolysis of glycogen with subsequent alkali treatment eliminates both glycogen and glucose and consequently no carbohydrate is found. Finally, treatment with alkali only destroys glucose, leaving the glycogen intact, which results in lower figures than without alkali treatment.

For routine determinations treatment with alkali for 1 hr. at 100° was adopted. After adding known amounts of glycogen to liver extracts the glycogen was fully recovered (Table 6).

Comparison of methods for estimation of glycogen in liver and muscle. The results of the method were compared with those of the technique described by Good *et al.* (1933). Two adjacent samples were taken from several livers, one sample extracted with TCA and the other one submitted to analysis according to Good *et al.* In the extracts glycogen was estimated with the anthrone reagent. The same experiments were performed with muscles of rats. To extract the glycogen from muscle, a 500 mg. sample was ground finely in a mortar with about 1 g. of purified sand and 10 ml. of 5% TCA. The sand and proteins were filtered off and the clear filtrate was used for analysis.

The data in Table 7 indicate a satisfactory agreement for liver but not for muscle, suggesting an incomplete extraction of glycogen from the latter. This assumption was tested as follows:

Two samples were taken from the same muscle; one extracted with TCA and the other one heated with alkali until a homogenous solution was obtained. From this solution the glycogen was precipitated by adding twice the volume of ethanol and the precipitate separated. The glycogen was dissolved in 10 ml. of TCA, yielding a solution comparable with that obtained by direct extraction of muscle. In the two extracts glycogen was estimated with the anthrone reagent (Table 8). Lower figures were obtained when the muscle was directly extracted with TCA than after liberation of the glycogen by destruction of the tissue with alkali. So apparently a considerable part of the glycogen in muscle is not extracted with TCA. Subsequent extractions of the protein residue with fresh TCA did not increase the yield of glycogen. However, the unextracted glycogen could be recovered after destruction of the protein residue with alkali.

In a similar series of experiments both the extractable and non-extractable part of the glycogen were estimated in the livers of rats, submitted to various treatments. The non-extractable glycogen left in the protein residue of the tissue after extraction with TCA was liberated by alkali destruction and separated by ethanol precipitation. In some cases the colour intensities of the glycogen-iodine complexes were determined as well. All results are

Table 5. *Influence of treatment with alkali on glucose and glycogen in extracts of incubated livers*

Results* (as % glucose equivalent of fresh wt. of liver)			
Acid hydrolysis previously		No acid hydrolysis	
Including KOH treatment	Without KOH treatment	Including KOH treatment	Without KOH treatment
0·01 ± 0·022	1·01 ± 0·424	0·33 ± 0·328	0·99 ± 0·471

* Each result is the average of eight experiments.

Table 6. *Recovery of glycogen added to liver extracts*

Estimation with anthrone reagent.

Added glycogen (mg./ml.)	Recovered glycogen (mg./ml.)	Recovery (%)
0·159	0·157	98·8
0·220	0·221	100·3
0·272	0·280	102·9
0·316	0·313	99·1
0·356	0·366	102·9
0·389	0·382	98·4
		Av. 100·4

Table 7. *Agreement between estimations with the anthrone reagent and the method of Good* et al. (1933)

	Glycogen (Mean % fresh wt. tissue)		
	With anthrone reagent	With Good's method	Mean difference
Liver	(10) 2·72	(10) 2·51	+0·21 ± 0·227
Muscle	(12) 0·34	(12) 0·52	−0·18 ± 0·050

The figures in parentheses give the numbers of observations. For the mean values of the glycogen amounts in liver and muscle no standard deviations have been calculated as the latter are only indicative of the variability between individual animals. For the comparison of the two methods only the differences between the data obtained with both methods in the same organ are relevant. The relatively greatest mean difference between the estimations in muscle is highly significant ($t_{11} = 12{\cdot}5$, $P < 0{\cdot}001$).

Table 8. *Determinations of glycogen in muscle of rats using trichloroacetic acid extraction or alkali-destruction*

	Glycogen found (Mean % fresh wt.)		
No. of observations	Glycogen extracted with TCA	Glycogen liberated by destruction of the tissue with alkali	Mean difference
18	0·42	0·61	0·19 ± 0·126

Statistical significance of mean difference $t_{17} = 6{\cdot}37$, $P < 0{\cdot}001$.

Table 9. *Extractable and non-extractable glycogen in the livers of rats and mice after various treatments*

Expt.	Glycogen fraction	No. of observations	Glycogen (% of fresh wt.)	Colour intensity*
1 Normal rats, not starved	Extractable	10	4·71±2·22	0·93±0·085
	Non-extractable	—	0·16±0·021	0·58±0·121
2 Normal rats, starved for 24 hr.	Extractable	10	0·04±0·024	—
	Non-extractable	—	0·03±0·016	—
3 Adrenalectomized rats, starved for 24 hr.	Extractable	5	0·06±0·045	—
	Non-extractable	—	0·01±0·007	—
4 Adrenalectomized rats, starved for 24 hr. and treated with cortisone†	Extractable	5	0·63±0·240	—
	Non-extractable	—	0·08±0·050	—
5 Adrenalectomized mice, starved for 24 hr. and treated with glucose‡	Extractable	5	0·46±0·274	0·87±0·224
	Non-extractable	—	0·10±0·049	0·50±0·174
6 Adrenalectomized mice, starved for 24 hr. and treated with cortisone and glucose‡	Extractable	5	1·98±0·541	1·26±0·263
	Non-extractable	—	0·13±0·32	0·64±0·131

* Statistical significance of differences between colour intensities of extractable and non-extractable glycogen fractions: Expt. 1: $t_9 = 7{\cdot}72$, $P < 0{\cdot}001$; Expt. 2: $t_7 = 3{\cdot}26$, $P < 0{\cdot}05$; Expt. 3: $t_7 = 9{\cdot}50$, $P < 0{\cdot}001$.

† After fasting for 16 hr. each rat received 5 doses of 0·18 mg. cortisone acetate in peanut oil subcutaneously.

‡ Treatment with glucose and cortisone as described by Venning, Kazmin & Bell (1946). Total doses for each animal: cortisone acetate 0·08 mg., glucose 35 mg.

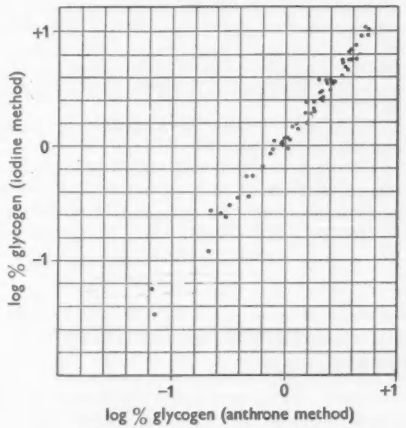

Fig. 3. Parallelism between the results of the anthrone and the iodine method. The logarithms of the percentage glycogen found with the two methods are plotted against each other.

summarized in Table 9. In the livers of normal animals the non-extractable fraction is only a small part of the total glycogen. The colour intensities with iodine at 650 mμ. of the non-extractable glycogen fractions are less than those of the extractable parts. Finally it can be seen that alterations in the glycogen content of liver, caused by starvation or treatment with cortisone acetate, are mainly present in the extractable fraction of the glycogen.

Comparison of anthrone and iodine methods. In many cases, the determination of the extractable glycogen will furnish sufficient information about the glycogen contents of the liver, and then the TCA extraction procedure may be adopted, with subsequent estimation by the iodine or anthrone method. A comparison between the anthrone and the iodine method was made using extracts obtained during a glycogenetic test. A close parallelism was found between both series of results as can be seen from Fig. 3 and the final results of the test proved to be nearly the same, irrespective of the method used for the estimation of glycogen. It may be noted that in the graph logarithms of the percentage are plotted. It could be shown that the logarithms of liver glycogen values in rats have a normal frequency distribution. So the logarithm rather than the actual percentage should be used for the statistical calculation of a test.

DISCUSSION

In the foregoing the usefulness of the iodine and anthrone methods has been demonstrated. Both methods are simple and much more appropriate for serial determinations than all other methods previously described. Nevertheless they have their limitations. The TCA extraction, which is used for both methods, is very convenient but a considerable part of the glycogen in the muscles of rats is not extracted with TCA. Consequently the TCA extraction cannot be recommended for the estimation of glycogen in muscle.

In liver the non-extractable glycogen fraction proved to be only a minor part of the total glycogen and alterations in the amount of glycogen in this tissue were found to be mainly associated with the extractable glycogen. For many purposes the determination of the extractable glycogen will give sufficient information about the amount of glycogen in a liver. The suitability of the extraction

procedure should be determined for other organs. If one wants to be sure that 100% of the glycogen present in any organ is determined, the usual hydrolysis of the tissue with strong alkali followed by precipitation of the glycogen remains the method of choice. Using the iodine reagent only approximate figures are obtained for the glycogen content of a liver extract. Nevertheless, this very convenient method may be useful for investigations where some accuracy may be sacrificed in order to make possible experiments with a large series of rats. For instance this was the case in our studies of the glycogenetic properties of adrenal steroids by estimation of liver glycogen, in which the experimental animals are treated following a standard procedure, and the results are always mutually compared. The results of these tests were hardly influenced by the variable colour intensities of the glycogen from different livers. However, if approximate values cannot be accepted the use of the anthrone method is indicated. Providing the lower saccharides are previously destroyed with alkali, more correct results are obtained, but the method is more elaborate than the estimation with iodine.

SUMMARY

1. Two methods are described for the determination of glycogen. In both procedures the glycogen is extracted from the tissue with trichloroacetic acid.

2. In these extracts glycogen can be estimated approximately by means of an iodine reagent. The colour intensity of the glycogen-iodine complex varies from one liver to another and is further influenced by the treatment of the animals.

3. More correct results are obtained if the glycogen in the extract is determined using an anthrone reagent, after treating the liver extracts with alkali which eliminates interfering glucose.

4. The application of the methods is briefly discussed.

I wish to express appreciation and thanks to Dr G. A. Overbeek for his advice and encouragement given during the course of this work and to Miss C. W. N. N. Schellekens and Mr J. J. de Koning for valuable technical assistance.

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The Use of Substances Depressing Antithrombin Activity in the Assay of Prothrombin

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Determination of the prothrombin activity of body fluids has become of clinical interest in connexion with the diagnosis of haemorrhagic disorders, and during treatment with anticoagulants. Most techniques for the determination of prothrombin are based on one or other of two principles. In both, prothrombin is converted into thrombin in the presence of optimum amounts of the other clotting factors. In one, the time interval required for clot formation in the specimen is taken as a measure of prothrombin activity; this is a direct method, and is referred to in the literature as the one-stage technique (Quick, 1942). In the other, the thrombin potency developed is estimated with an added substrate containing fibrinogen; this is an indirect method often called the two-stage technique (Warner, Brinkhous & Smith, 1936).

In the direct method a fibrin clot appears at a certain time corresponding to the formation of a certain concentration of thrombin. The rate of thrombin formation depends upon the concentration of prothrombin and the activity of accelerating and inhibiting factors. The direct method is useful in determining the haemostatic condition of a patient, but it does not indicate the actual prothrombin concentration of the specimen.

In the direct method, fibrin formation takes place at a very low thrombin concentration, which is

normally only a small fraction of the potential total thrombin yield. The bulk of thrombin production will therefore occur after coagulation has taken place. Most biological fluids which contain the clotting components can also inactivate thrombin. Therefore the yield of thrombin is determined by the balance between formation and inactivation. It is apparent that this will vary with the concentration of prothrombin and the antithrombic potency of the specimen.

On the other hand, in the indirect method the total yield of thrombin obtainable from the prothrombin-containing preparation is determined, and this is not dependent on the rate of thrombin formation. The indirect method is less suitable for the detection of a haemorrhagic condition, but gives an indication of the quantitative relation between prothrombin activity and its concentration. Because of the different principles involved the results obtained by the two methods do not always agree. In the most widely used indirect process for the determination of prothrombin devised by Warner *et al.* (1936), the activity of antithrombin is reduced by dilution of the specimen and by addition of gum acacia (Seegers & Smith, 1942). Sternberger (1947) has found that the presence of 25% ethanol ensures thrombin stability. The following experiments indicate that a number of phenols which accelerate the thrombin fibrinogen reaction have also the property of depressing antithrombin activity. A prothrombin assay including such substances is presented.

EXPERIMENTAL

Materials

Venous blood from humans of both sexes, aged 2–70 years, was obtained at least 2 hr. after a meal. Animals were anaesthetized with Nembutal and ether, and blood was obtained from guinea pigs by heart puncture, and from rabbits by cannulating the carotid artery. Blood (9 vol.) was mixed quickly with either 1 vol. of 0·1 M sodium oxalate or with 1 vol. of 0·13 M trisodium citrate. Cell-free plasma was obtained after centrifuging at 2300 *g* at 6° for 20 min. Both cell and plasma volume were measured. The plasma specimens were kept at 6° until the test was carried out, which was not more than 6 hr. after collection. Human serum was obtained from blood collected without any addition. It was separated from the clot after at least 24 hr. storage at 20°. Usually serum from two donors was pooled.

$BaCO_3$ was freshly prepared according to Fantl & Nance (1947); $BaSO_4$ was prepared from equivalent amounts of $Ba(OH)_2$ and dil. H_2SO_4, and washed with distilled water until neutral. The wet suspensions of the barium salts were used for not more than 1 week after preparation.

Prothrombin-free plasma, which will be called Ba-plasma, was obtained by mixing suspensions containing 10–20 mg. $BaSO_4$, or $BaCO_3$/ml. of oxalated plasma, and frequent stirring in ice water for 30 min.

Thromboplastin preparations were made from human or rabbit brain, which was thoroughly rinsed with 0·15 M-NaCl and dehydrated with acetone according to Quick (1942). The dry powder was kept in evacuated ampoules in the refrigerator. On the day of use, a 6% suspension in 0·15 M-NaCl was prepared, kept at 36° with occasional stirring for 1 hr., and then on ice for 1–2 hr. The sediment-free fluid was used.

Isolation of the euglobulin fraction from plasma and its conversion into thrombin was carried out essentially according to Mellanby (1909). Bovine thrombin was a commercial product.

Dilutions were made from a 0·25 M-$CaCl_2$ stock solution, preserved with 1×10^{-4} part of merthiolate.

Human fibrinogen was prepared by the process of Kekwick, Mackay & Record (1946).

A commercial preparation of gum acacia was purified according to Seegers & Smith (1942). Phenol, *m*- and *p*-cresol, pyrocatechol, resorcinol and quinol were commercial products of analytical grade.

The veronal buffer of Michaelis (1931) was used.

Procedure

In the determination of the depression of antithrombic activity of human serum by phenols, the phenol to be tested was dissolved to 0·036 M in 1·5 ml. of a solution containing 0·01 M-$CaCl_2$, 0·06 M-NaCl; this solution contained 0·3 ml. of veronal buffer, pH 7·4. To this mixture were added 0·1 ml. human serum and finally 0·2 ml. of a human or bovine thrombin preparation containing 0·15 M-NaCl. In control experiments the phenols were replaced by NaCl. From the reaction mixtures, 0·2 ml. samples were removed at known intervals, and blown into a mixture of 0·2 ml. human Ba-plasma and 0·05 ml. of 0·18 M solution of the phenol to be tested. The reactions were carried out in a water bath maintained at 28°±1°. The clotting times were measured and converted into thrombin units (see below).

RESULTS

Choice of phenol. The results in Fig. 1 show the quantitative differences among the phenols with regard to their ability to stabilize thrombin in the presence of human serum. *p*-Cresol and *m*-cresol were more effective than quinol. During an incubation time of 3 min., approximately 10% thrombin disappeared in the presence of pyrocatechol, phenol or resorcinol, whilst in the absence of the phenols, more than 40% thrombin activity disappeared. The antithrombic activity of human serum varied. In several other experiments the loss of thrombin could be prevented by phenols for a period of 5–10 min. A comparison between the activity of gum acacia, which is used in the prothrombin assay of Warner *et al.* (1936), and pyrocatechol was carried out, and the results are given in Fig. 2.

The results given in Fig. 2 show two facts. First, the clotting time in the presence of pyrocatechol corresponded to 1·2 units of thrombin, whilst 0·85 unit seemed to be present with gum acacia (see Fig. 3). Secondly, pyrocatechol showed greater antithrombin depressing activity than gum acacia. These two properties appeared to be of advantage

in an assay of prothrombin. Satisfactory results could be obtained in the presence of 0·03 M *m*-cresol with plasma; however, isolated prothrombin preparations gave a reduced yield of thrombin. Pyrocatechol did not interfere with clotting reactions if used in concentrations between 0·03 and 0·04 M.

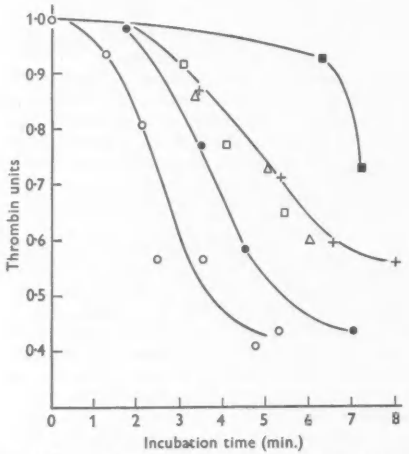

Fig. 1. Depression of antithrombic activity of human serum by phenols. ○—○, 0·1 ml. serum plus thrombin; ●—●, 0·1 ml. serum plus quinol; □—□, 0·1 ml. serum plus resorcinol; +—+, 0·1 ml. serum plus pyrocatechol; △—△, 0·1 ml. serum plus phenol; ■—■, 0·1 ml. serum plus *m*- or *p*-cresol.

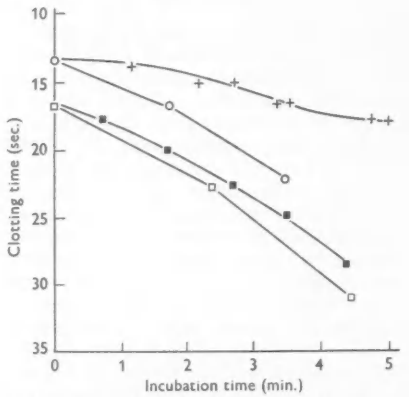

Fig. 2. Depression of antithrombic activity of human serum by pyrocatechol 0·03 M or by gum acacia 2·5%. Pyrocatechol experiments: ○—○, serum plus thrombin; +—+, serum plus pyrocatechol. Gum acacia experiments: □—□, serum plus thrombin; ■—■, serum plus gum acacia. Experimental conditions similar to those given in Fig. 1.

Definition of prothrombin units. The relationship between clotting time and thrombin activity was established with varying concentrations of a thrombin preparation under similar conditions to the assay of prothrombin, except that the addition of the prothrombin-containing material was omitted. One unit (PTU) of prothrombin is here defined as that amount of prothrombin present in 0·2 ml. fluid containing 0·036 M pyrocatechol which, when converted into thrombin and added to a mixture of 0·2 ml. human Ba-plasma and 0·05 ml. of 0·18 M pyrocatechol in veronal buffer, pH 7·4, produces a clot in 15 sec. at 28°. One unit of prothrombin corresponds to 1 unit of thrombin. In the method of Warner *et al.* (1936) a different unit of thrombin is used. A definition of it is quoted by Ware & Seegers (1949) as follows: 'A thrombin unit is that amount of thrombin which will clot 1 ml. of standardized fibrinogen solution in 15 sec., under the conditions imposed for the two-stage analysis'. The test is carried out in the presence of 2·5% gum acacia. This unit will be referred to here as gum acacia thrombin unit (GTU). On a volume basis one PTU corresponds to 0·45 GTU. In both definitions 1 unit is based on a clotting time of 15 sec., but a given clotting time in the range of 9–15 sec. indicates approximately 1·4 times greater thrombin potency in the presence of 0·036 M pyrocatechol than it does in the presence of 2·5% gum acacia. Thus, to convert PTU into GTU, it is necessary to divide by $1{\cdot}4 \times 2{\cdot}22$.

It was thought advisable to introduce the PTU for the following reason. In the method of Warner *et al.* (1936), a 1% fibrinogen solution is diluted with the thrombin-containing material to 0·2%. Such concentrations cannot be obtained when normal human plasma is used as fibrinogen solution; but it is desirable to carry out the thrombin assay with

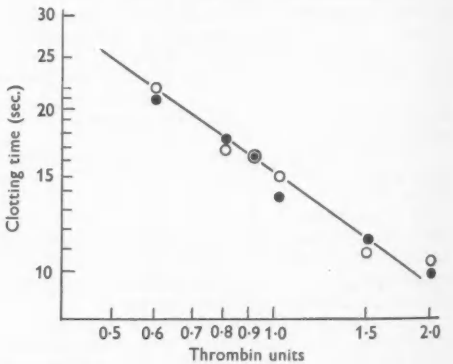

Fig. 3. Relationship between thrombin concentration and clotting time. ●—●, pyrocatechol; ○—○, gum acacia. Experimental conditions similar to those given in Fig. 1.

fibrinogen concentrations above 0·1% because a small variation of fibrinogen concentration in this region has little influence on the thrombin clotting time (Deutsch & Gerarde, 1946; Fantl & Nance, 1948). The relationship between the $\log_{10}$ of the thrombin concentration, and the $\log_{10}$ of the clotting time is given in Fig. 3. For practical purposes it is convenient to draw a line from one point corresponding to 1·9 units and 10 sec. clotting time to 0·5 unit and 25 sec. Using this graph, an error not greater than 10% from the calculated value will be found. This is sufficiently accurate because duplicates of clotting times cannot be reproduced with greater precision.

Assay of prothrombin. For the assay of prothrombin, the following reaction mixture was prepared: into Pyrex tubes (9–10 mm. diameter, 80 mm. length) were measured 1·5 ml. of a mixture consisting of: 0·15 ml. of the thromboplastin preparation, 0·6 ml. 0·025 M-$CaCl_2$, 0·45 ml. 0·15 M-NaCl, 0·3 ml. 0·18 M pyrocatechol in veronal buffer pH 7·4; to this was added 0·3 ml. of the material to be tested.

At known periods beginning 1–2 min. after the last addition, 0·2 ml. was blown into a mixture of 0·2 ml. human Ba-plasma and 0·05 ml. 0·18 M pyrocatechol, and the clotting times were determined. The reactions were carried out in a water bath at 28±1°. Three successive samples which gave identical clotting times indicated a maximum yield of thrombin.

To obtain reliable results it was necessary to use very active thromboplastin preparations, and the optimum concentration of brain extract to give the shortest clotting time had to be established in preliminary tests. The concentration of the material to be tested depended on its prothrombin activity.

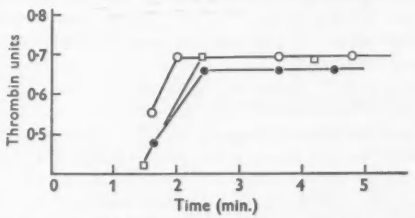

Fig. 4. Prothrombin assays in human oxalated plasma with and without defibrination. □—□, plasma not defibrinated; ○—○ plasma defibrinated with 2 units of bovine thrombin; ●—●, plasma with 1 unit of bovine thrombin.

Samples of reaction mixtures containing 0·015 ml. of normal oxalated or citrated human plasma should clot in 15–20 sec. after 2–5 min. incubation. The assays were carried out with either human- or rabbit-brain extract. This did not seem to make any difference to the result. In the recommended assay of prothrombin, fibrinogen is not removed from the test fluid. Some workers using the technique of Warner *et al.* (1936) defibrinate the specimen, while others omit this step (Herbert, 1940). Prothrombin assays were carried out on specimens with and without defibrination. The results in Fig. 4 show that the presence of fibrinogen or fibrin had little influence on the rate of thrombin formation and thrombin yield. Therefore it seems unnecessary to remove fibrinogen prior to the assay of prothrombin.

A further simplification in the proposed assay is the use of human Ba-plasma instead of isolated fibrinogen. In order to test the validity of this substitution, prothrombin assays were carried out simultaneously with human Ba-plasma and human fibrinogen, and also with rabbit Ba-plasma.

It is apparent that the results (Table 1) with human Ba-plasma or isolated human fibrinogen are not significantly different, and justify the replacement of fibrinogen by human Ba-plasma in the prothrombin assay. The results also indicate that the sensitivity of thrombin to rabbit's Ba-plasma is somewhat less than to human Ba-plasma. Finally, with regard to the optimum Ca ion concentration, it was observed that the prothrombin results did not vary significantly when the assays were carried out in reaction mixtures containing between 0·017 M- and 0·0085 M-$CaCl_2$.

Variation of prothrombin activity in normal mammalian plasma. It appeared of interest to determine, by the assay method described, prothrombin activity in a number of human and animal plasma specimens. The 126 results recorded in the histogram (Fig. 5) were obtained with either pooled or single plasma specimens from 159 human donors, who on clinical grounds were expected to have a normal prothrombin level. 94% of the results are between 375 and 575 units (PTU) of prothrombin/ml. oxalated plasma. This corresponds to 470–720 (PTU)/ml. of native plasma. Results obtained by previous workers using the method of Warner *et al.* (1936) are quoted in

Table 1. *Prothrombin assays of oxalated plasma with different sources of fibrinogen*

Results are given in units PTU/ml. oxalated plasma

	Plasma							
Source of fibrinogen	Bu*	Bl	Ma	Ea	Ro	Gr	Be	Rabbits
Human Ba-plasma	600	400	380	460	470	430	450	380–450
Human fibrinogen	550	370	340	—	—	—	—	—
Rabbit Ba-plasma	—	—	—	410	380	380	330	310

* Bu...Be refer to the name of the donor.

Table 2. The apparently higher results obtained with the pyrocatechol technique than with the gum acacia technique are due to the difference in the definition of the thrombin units.

As an essential difference between the technique devised by Warner *et al.* (1936) and the one suggested here is that gum acacia is replaced by pyrocatechol; it was necessary to compare the results obtained from assays using these two substances. The results given in Table 3 indicate that prothrombin assays carried out in the presence of pyrocatechol gave 20–60 % higher results than in the presence of gum acacia.

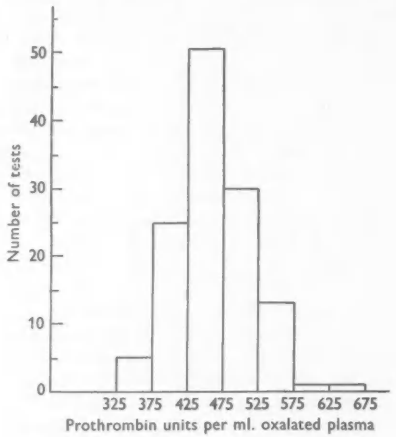

Fig. 5. Prothrombin assays in 126 specimens of human oxalated plasma.

Table 2. *Results of prothrombin assays on normal mammalian blood plasma*

Figures are given in units/ml. of native plasma. Number of specimens in brackets.

Man	(45)	244–452 GTU	Mann *et al.* (1948)
	—	300–350 GTU	Ware & Seegers (1949)
	(126)	470–720 PTU	Present work
Rabbit	—	220–270 GTU	Murphy & Seegers (1948)
	(5)	450–670 PTU	Present work
Guinea pig	—	210–250 GTU	Murphy & Seegers (1948)
	(2)	310–330 PTU	Present work

Table 3. *Prothrombin assays of citrated and oxalated human plasmas and a citrated euglobulin fraction of human plasma*

The results are given as units/ml.

Source	Pyrocatechol (0·03 M)	Gum acacia (2·5 %)
Hu. citrated	470	380
Hu. oxalated	440	290
Lo. citrated	450	380
Lo. oxalated	430	300
Go. citrated	300	180
No. oxalated	420	270
Euglobulin citrated	660	480

Occasionally prothrombin determinations have to be carried out in plasma, serum or isolated fractions, in which it may be necessary to add prothrombin accelerator to obtain the maximum thrombin yield. The addition of 0·1 ml. of a 20 % Ba-plasma dilution to the reaction mixture has been found adequate for this purpose.

Prothrombin has been assayed with venous and capillary blood. In the latter case, 0·027 ml. blood was taken from a finger or heel puncture. The results obtained from venous and capillary blood were identical.

DISCUSSION

The experimental results presented show that the inactivation of thrombin by serum can be delayed and partly depressed by several phenols. Provided that these substances have no influence on the formation of thrombin, they can be used in the assay of prothrombin. One of the phenols used, pyrocatechol, belongs to a series of compounds which accelerates the thrombin fibrinogen reaction. Gerber & Blanchard (1945) assume this to be due to a catalytic influence of phenols on thrombin, and so the addition of pyrocatechol to a clotting system would give erroneously high values of the thrombin content. However, it was shown recently (Fantl & Ebbels, 1953) that pyrocatechol affects the surface structure of fibrinogen, making it more sensitive to the action of thrombin.

It is generally agreed that the method of Warner *et al.* (1936) gives absolute prothrombin values. Modifications of that method using different buffers have been suggested by Rieben (1947), Hurn & Mann (1947), Mawson (1949) and Herbert (1940). Ware & Seegers (1949) add prothrombin accelerator to ensure complete conversion of prothrombin into thrombin. In these techniques, isolated fibrinogen is used as the indicator of thrombin activity. As the variable reactivity and limited stability of most fibrinogen preparations are difficulties in these techniques, Sternberger (1947) has used plasma instead of fibrinogen, and ethanol as antithrombin depressing agent. The disadvantage of this procedure is that in the presence of ethanol, thrombin produces a granular fibrin precipitate which is not easily detected.

The present experiments indicate that it is unnecessary to defibrinate plasma specimens prior to the test, and that fibrinogen can be replaced by human Ba-plasma. Rabbit Ba-plasma is less reactive towards thrombin. Pyrocatechol as an antithrombic depressing agent has its limitations. In the suggested assay, plasma with a normal prothrombin concentration is diluted 120 times. It has

often been found that the thrombin formed in the presence of pyrocatechol was stable for periods up to 20 min., whilst in its absence, loss occurred after 4–5 min. On the other hand, in the case of a prothrombin concentration of approximately 20% of the normal value, or less, where the plasma is diluted 12 times, significant losses of thrombin occur even in the presence of pyrocatechol after 3 min. incubation. Despite this, a satisfactory assay can be carried out since complete conversion of prothrombin into thrombin occurs in that period. It is preferable to carry out the assay of prothrombin with citrated plasma, because calcium oxalate adsorbs prothrombin (Menghini, 1949). The assay both in a macro- and in a micro-form has been carried out in blood, plasma, and serum of man and other mammals and also in isolated plasma fractions.

SUMMARY

1. Phenols depress the antithrombic activity of serum.

2. This property, as well as the accelerating effect of phenols on the thrombin fibrinogen reaction, is utilized in a prothrombin assay.

3. Results of prothrombin assays in mammalian plasma in the presence of pyrocatechol are presented.

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Creatine Phosphokinase: Assay and Application for the Micro-determination of the Adenine Nucleotides

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The determination of creatine phosphokinase activity has been complicated by the fact that although creatine phosphate is extremely labile in acid solution, the assays of this enzyme which have been described in the literature (e.g. Banga, 1943; Askonas, 1951; Narayanaswami, 1952) have at some stage usually involved exposure to a pH well below 7. Creatine phosphate breakdown during deproteinization with trichloroacetic acid can be minimized by working at low temperature, but the subsequent determination of this substance by methods involving phosphate analysis presents difficulties when the system contains other labile organophosphates or free phosphate. Usually creatine phosphate has been determined by phosphate analysis after fractionation (Meyerhof, 1930; LePage & Umbreit, 1945), or after hydrolysis with acid molybdate (Fiske & Subbarow, 1929). The latter method is not entirely satisfactory, however, as a number of workers, particularly Weil-Malherbe & Green (1951), have indicated that under the acid molybdate conditions, creatine phosphate may not be the only labile organophosphate. When creatine phosphate is estimated in systems containing

relatively higher concentrations of adenosine diphosphate or adenosine triphosphate the errors in the acid molybdate method may be considerable due to the slow breakdown of the latter substances. The method of Lowry & Lopez (1946) permits the estimation of phosphate in the presence of acid-labile organophosphates but even under these conditions there is a slow breakdown of creatine phosphate.

The estimation of creatine rather than phosphate as a means of following the reaction offers the advantage that determinations can be made under alkaline conditions, in which creatine phosphate is stable, by the method of Eggleton, Elsden & Gough (1943). In general this method has not been widely used, but Ennor & Stocken (1948) have investigated some of the sources of error while Ennor & Rosenberg (1952) have adapted it for the estimation of the creatine phosphate content of tissues.

In the present paper details are given of a method for the assay of creatine phosphokinase activity by following the liberation of creatine. The interactions between the creatine phosphokinase system and the myokinase and hexokinase systems, respectively, have been studied and in particular the latter interaction has been adapted for the colorimetric estimation of extremely low concentrations of adenosine polyphosphates.

EXPERIMENTAL

Methods

Creatine phosphokinase. Minced rabbit muscle was treated for 30 sec. in a Waring Blendor with 3 vol. of a medium containing 0·025 M-KCl and 0·039 M borate buffer, pH 7·1. This medium extracts the minimum of protein from the myofibrils (Perry, 1953). After being stirred for 30 min. the extract was filtered through muslin, the residue re-extracted with 2 vol. of borate:KCl and again passed through muslin. The combined filtrates were centrifuged for 15 min. at 600 *g* and the preparation of creatine phosphokinase carried out either as described by Askonas (1951), employing butanol treatment followed by propanol fractionation, or by the Banga (1943) procedure up to the fourth stage. Preparations were finally dialysed with stirring for 14 hr. against glass-distilled water, and in some cases freeze-dried. Freeze-dried preparations were dissolved in glass-distilled water as they were required for use.

Crystalline yeast hexokinase. Hexokinase was prepared by Mr E. J. Morgan after Kunitz & Macdonald (1943) and stored at $-15°$ as an $(NH_4)_2SO_4$ paste. For use the paste was dissolved in 1% (w/v) glucose and dialysed against 1% glucose for 24 hr.

Myokinase. Myokinase was prepared by the method of Kalckar (1947) and was stored at 0° as an $(NH_4)_2SO_4$ paste.

Muscle acetone-powder extracts. Dialysed extracts were prepared as described in the following paper (Perry, 1954).

Hexokinase activity. Hexokinase activity was assayed by following the disappearance of 10 min. P, i.e. inorganic P liberated on heating for 10 min. in N acid at 100°, when hexokinase was added to a reaction mixture containing 0·005 M-$MgSO_4$, 60 μmoles glucose and 12–15 μmoles adenosine triphosphate (ATP). The total volume was 2 ml. and incubation was at 30°. One unit of hexokinase activity was defined as that amount of enzyme which would transfer 1 μmole of phosphate from ATP to glucose in 5 min.

Deproteinization. After incubation in phosphate buffer deproteinization was carried out using the alkaline reagents of Somogyi (1945). Usually after inactivation of the enzyme 0·2 ml. of 0·3 N-$Ba(OH)_2$ was added followed by 0·2 ml. of the 5% $ZnSO_4$, $7H_2O$ reagent. When aminotrishydroxymethylmethane (Tris) buffer (Gomori, 1946), was used it was necessary to add NaOH to the $Ba(OH)_2$ solution so that the deproteinized incubation mixture was just pink to phenolphthalein.

Creatine. Creatine as supplied by British Drug Houses Ltd. was recrystallized from hot water as the monohydrate.

Creatine phosphate. Sodium creatine phosphate hexahydrate was prepared synthetically by Mr E. J. Morgan using the method of Zeile & Fawaz (1938). It was stored in the cold over $CaCl_2$, and 0·1 M solutions were made up as required. The creatine blank of these solutions was very low and did not increase appreciably during storage at $-15°$ for periods up to 2 months.

Adenosine triphosphate. ATP was prepared by a method similar to that described by Needham (1942). The Ba salt was converted into the Na salt as described by Chappell & Perry (1953).

Adenosine diphosphate. ADP was prepared by Mr E. J. Morgan from ATP as described by Bailey (1942). It was stored as the Ba salt and converted into the Na salt by the same method as used for ATP.

Creatine estimation. The reagents were prepared and estimations carried out as recommended by Eggleton *et al.* (1943). The stock alkali was stored at 30° since at room temperature crystallization tended to occur. Colour intensities were measured using filter 303 in an EEL photoelectric colorimeter (Evans Electroselenium Ltd.). Some measurements were also made on a Beckman apparatus at 520 mμ.

Phenylmercuric acetate. This reagent was supplied by Hopkin & Williams. It was used in 0·05 M solution, made by dissolving 0·34 g. in 10 ml. dioxan and adding 10 ml. glass-distilled water. The *p*-chloromercuribenzoic acid used in some experiments was synthesized by Mr B. R. Slater by the method of Whitmore & Woodward (1932).

Adenylic acid. Dr A. L. Lehninger kindly provided the sample of adenylic acid (Bischof Ltd.) used in this investigation. No ATP or ADP could be detected in this sample by the chromatographic method of Eggleston & Hems (1952).

RESULTS

Essential requirements of the method were that the enzyme reaction should be stopped rapidly and effectively, and deproteinization be accomplished without the pH falling below 7 at any stage. The barium hydroxide and zinc sulphate reagents of Somogyi (1945), however, did not destroy completely the enzyme activity and were therefore employed after the enzyme had been inactivated as described below.

As there is considerable evidence suggesting that free sulphydryl groups are essential for creatine phosphokinase activity (Lehmann & Pollack, 1942;

Askonas, 1951; Bailey & Marsh, 1952), organomercury compounds were considered likely substances to inactivate this enzyme effectively. In particular *p*-chloromercuribenzoate and phenylmercuric acetate were examined and found to be highly satisfactory when used at a final concentration of 0·01 M. *p*-Chloromercuribenzoate is conveniently soluble in alkali, but unlike phenylmercuric acetate was not readily available in the quantities required. Phenylmercuric acetate is only sparingly soluble in water but was used as a 0·05 M solution in 50 % (v/v) aqueous dioxan in which it was readily soluble. It was apparent from many experiments, an example of one type being given in Table 1, that at the concentration used phenylmercuric acetate completely and instantaneously inactivated the enzyme.

Table 1. *Inactivation of creatine phosphokinase by phenylmercuric acetate*

Each reaction mixture contained 0·005 M-$MgSO_4$, 5 μmoles ADP, 10 μmoles creatine phosphate, 0·05 M potassium phosphate buffer, pH 7·4; 0·5 ml. 0·05 M phenylmercuric acetate was then added, followed by the enzyme. The total volume was 2·5 ml. The tubes were then incubated for 75 min. at 30°. In the control tube the enzyme was added at zero time and phenylmercuric acetate at the end of the incubation period.

Creatine phosphokinase (ml.)	Time of addition of phenylmercuric acetate	Creatine liberated (μg.)
0	—	0
0·2	Zero time	2
0·4	Zero time	1
0·2	After 75 min.	300

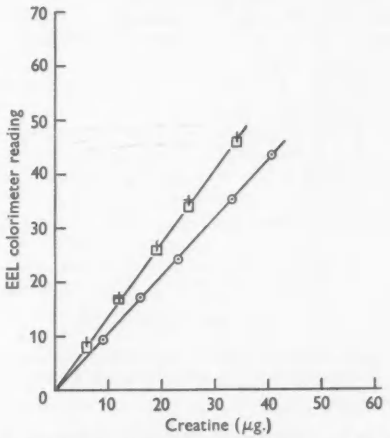

Fig. 1. The recovery of creatine from incubation mixtures. Varying amounts of a standard creatine solution were added to a typical reaction medium, which contained 0·005 M-$MgSO_4$, 10 μmoles creatine phosphate, 5 μmoles ADP, 0·36 mg. serum albumin, and either 0·1 M potassium phosphate buffer, pH 7·4, or 0·05 M Tris buffer, pH 7·4; total volume, 2·0 ml. To this medium 0·5 ml. 0·05 M phenylmercuric acetate was added, and the deproteinization method appropriate to the buffer was carried out. Creatine was then estimated in the deproteinized solutions. +, direct estimation of creatine; □, recovery with phosphate buffer; ⊙, recovery with Tris buffer.

An additional advantage of using organomercurials to inactivate the enzyme was that they also effectively removed sulphydryl compounds (cysteine was added to the incubation medium in some assays) which might have inhibited the colour formation in subsequent creatine estimations (Ennor & Stocken, 1948).

Some care was necessary in the choice of buffer for the enzyme assays because the development of colour in the creatine estimation was inhibited by buffers such as glycine, glycylglycine and veronal. Phosphate and Tris buffers were found to be suitable. With the usual incubation mixture containing phosphate buffer to which phenylmercuric acetate was later added followed by alkaline deproteinization, recovery was quantitative; when Tris was used, however, the recovery was only about 80 %, but the results were reproducible (Fig. 1). Similar results were obtained when enzyme tests were carried out in 0·01 M cysteine or over a wide range of magnesium sulphate concentrations. Doubling the phenylmercuric acetate concentration or increasing the dioxan present during the colorimetric estimation to 20 % had no effect on creatine recovery. Finally the procedure given below was adopted.

Measurement of creatine phosphokinase activity

The incubation mixture contained 0·05 M phosphate or Tris buffer, pH 7·4, 0·005 M-$MgCl_2$ or $MgSO_4$, 0·002–0·005 M ADP, 0·01 M cysteine (freshly neutralized before use), 0·005 M creatine phosphate, enzyme solution and water to 2 ml. Incubation was carried out at 30°. The enzyme solution was diluted appropriately just before assay and added to the incubation tube, which was then allowed to equilibrate at 30° for 4 min. The reaction was started by the addition of the creatine phosphate and stopped after 3–5 min. by the addition of 0·5 ml. of 0·05 M phenylmercuric acetate in 50 % (v/v) aqueous dioxan. Creatine liberation was determined at several concentrations of enzyme. The results were plotted graphically, as in Fig. 2, and the activity of the preparation calculated from the slope.

Aqueous extracts of muscle acetone powder and purified enzyme preparations obtained by the methods of Banga (1943) and Askonas (1951), respectively, had extremely high creatine phosphokinase activity and needed to be diluted several hundred times before they could be satisfactorily

assayed. To avoid spontaneous inactivation, cysteine was an essential component of the incubation medium when these dilute preparations were used. This feature is well illustrated in Fig. 2 which compares the effect of enzyme concentration with and without cysteine present in the incubation mixture, on the amount of creatine liberated in a system containing ADP, creatine phosphate and Mg^{2+}. Fig. 3 shows that magnesium ion is an essential component of the system; in its absence the activity was only about 3% of that obtained in the presence of 0·005 M-$MgSO_4$, the optimum concentration for activation. Increasing the concentration of ADP resulted in an increase in the rate of creatine liberation up to a maximum at approximately 0·002 M. Further increase in ADP concentration produced a very slight fall in activity (Fig. 4).

Under standard incubation conditions the method gave reproducible values for various creatine phosphokinase preparations; this is illustrated in Table 2 which contains the results of five independent determinations, carried out over a period of 13 days, of the creatine phosphokinase activity of an aged dialysed aqueous extract of muscle acetone powder.

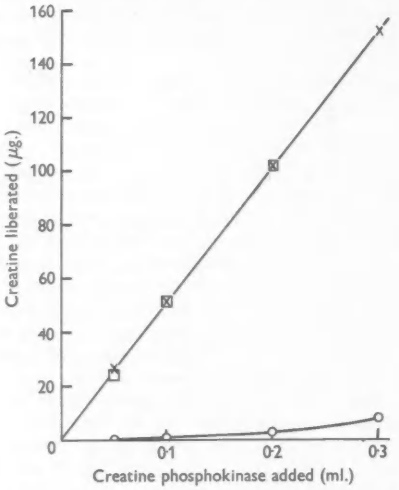

Fig. 2. The effect of enzyme concentration on creatine phosphokinase activity. The complete incubation mixture contained 0·005 M-$MgSO_4$, 5 μmoles ADP, 10 μmoles creatine phosphate, 0·01 M cysteine, 0·05 M Tris buffer, pH 7·4. After temperature equilibration the enzyme was added and incubated for 5 min. at 30°. The total volume was 2·0 ml. ×, □, duplicate determinations with the complete system; ○, without cysteine.

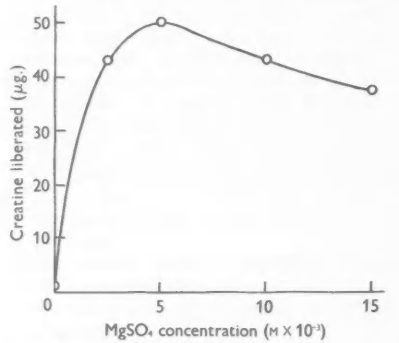

Fig. 3. The effect of Mg^{2+} concentration on creatine phosphokinase activity. The conditions were as described in Fig. 2.

Interaction of creatine phosphokinase and hexokinase: estimation of adenine nucleotides

When hexokinase and glucose are added to the creatine phosphokinase enzyme system the following sequence of reactions take place:

$$\text{Creatine phosphate} + \text{ADP} \overset{Mg^{2+}}{\rightleftharpoons} \text{creatine} + \text{ATP}. \quad (1)$$

$$\text{ATP} + \text{glucose} \overset{Mg^{2+}}{\longrightarrow} \text{ADP} + \text{glucose 6-phosphate}. \quad (2)$$

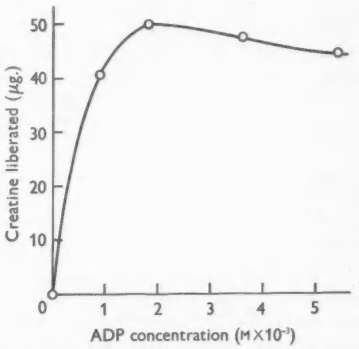

Fig. 4. The effect of ADP concentration on creatine phosphokinase activity. The conditions were as described in Fig. 2.

Table 2. *Creatine phosphokinase activity of aged dialysed aqueous extract of muscle acetone powder*

Incubation carried out at 30° in presence of 0·005 M ADP and 0·005 M-$MgCl_2$.

Date	Activity (μg. creatine liberated/hr./ml. enzyme)	Dilution used for assay
30. i. 53	161 000	1 in 500
31. i. 53	173 000	1 in 500
3. ii. 53	161 000	1 in 1000
6. ii. 53	171 000	1 in 1000
11. ii. 53	164 000	1 in 1000

In the presence of excess hexokinase very little ATP will accumulate in the system for as rapidly as it is formed by the Lohmann reaction it will be reconverted to ADP. Thus when both ADP and hexokinase are present in excess relative to creatine phosphokinase, the amount of creatine liberated is directly proportional to the activity of the creatine phosphokinase. This was demonstrated experimentally under conditions in which doubling the amount of hexokinase present or halving the concentration of ADP did not appreciably affect the rate of creatine production at any of the concentrations of creatine phosphokinase used.

It might also be expected from the equations (1) and (2) that ADP would become the limiting factor in the presence of excess of creatine phosphokinase and a given hexokinase concentration. The results shown in Fig. 5 indicate that this indeed is the case. Purified enzyme preparations (Askonas, 1951) were used in this particular system and the amount of creatine liberated was in linear relation to the amount of added ADP. No creatine was liberated in the absence of added ADP.

A feature of this system was its extreme sensitivity to low concentrations of ADP, e.g. in the experiment illustrated in Fig. 6, 0·0056 μmole ADP (i.e. 2·4 μg.) gave rise to 47 μg. creatine after 6 min. incubation. ATP behaved in a similar manner and when compared on a molar basis with ADP in the same system it was equally effective in catalysing the liberation of creatine (Fig. 6).

Purified creatine phosphokinase is specific for ADP and will not phosphorylate adenylic acid (Banga, 1943), and hence the latter substance did not catalyse the liberation of creatine at any of the concentrations tried. In the presence of myokinase, adenylic acid was almost as efficient as ADP, on a molar basis, in catalysing the liberation of creatine (Fig. 5). This system is of particular interest in that if it contained only creatine phosphokinase, hexokinase, myokinase, creatine phosphate, adenylic acid and glucose there would be no production of ADP, which is essential for the liberation of creatine by the creatine phosphokinase reaction. For the conversion of adenylic acid (AMP) into ADP by the myokinase reaction ATP is necessary (reaction 3).

$$\text{AMP} + \text{ATP} \rightleftharpoons 2\,\text{ADP}, \qquad (3)$$

$$\text{ADP} + \text{creatine phosphate} \rightleftharpoons \text{ATP} + \text{creatine}, \qquad (1)$$

$$\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose 6-phosphate}. \qquad (2)$$

Only a very small amount of ATP relative to the adenylic acid added need be present for the reactions (3) and (1) working together to bring about the autocatalytic conversion of adenylic acid to ADP. In the conditions employed in the experiment illustrated in Fig. 5 the hexokinase is tending to compete with the autocatalytic action of reactions (1) and (3) by reconverting ATP into ADP, but the myokinase reaction can produce two molecules of ADP per molecule of ATP, whereas the hexokinase reaction can yield only one. The net result in presence of the creatine phosphokinase system is that there will be an autocatalytic production of ATP. This ensures that when excess of myokinase is present the adenylic acid will exist virtually as ADP. That this is so is shown by Fig. 5 where adenylic acid is practically as efficient as ADP or ATP in liberating creatine under the particular experimental conditions. This means that the

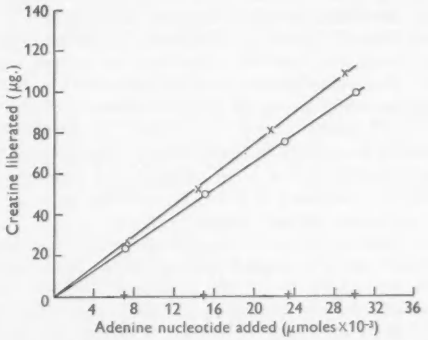

Fig. 5. The effect of varying the ADP and adenylic acid concentration in the presence of creatine phosphokinase (Askonas, 1951), hexokinase and myokinase. The incubation medium contained 0·05 M Tris, 0·005 M-$MgSO_4$, 10 μmoles creatine phosphate, 50 μmoles glucose, 6 units hexokinase. The total volume was 2·0 ml. The reaction was started by adding creatine phosphokinase and stopped after 5 min. at 30°. ×, ADP concn. varied; +, adenylic acid concn. varied; ○, adenylic acid concn. varied in the presence of myokinase.

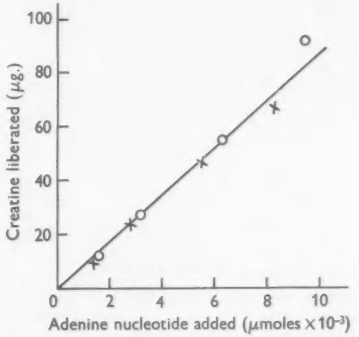

Fig. 6. Liberation of creatine by ADP and ATP in creatine phosphokinase–hexokinase system. Incubation medium contained 0·05 M phosphate buffer, pH 7·4, 0·005 M-$MgCl_2$, 10 μmoles creatine phosphate and 60 μmoles glucose. 6 min. incubation at 30°. ○, ATP; ×, ADP.

enzyme preparations must contain sufficient ADP or ATP to prime the myokinase reaction, though the concentrations of ADP or ATP needed are much lower than that of the added adenylic acid.

It is of interest to note that similar results with ATP and ADP on the one hand and adenylic acid on the other can be obtained if hexokinase is replaced by a magnesium-activated ATPase as is found in isolated myofibrils (see Perry, 1954).

Creatine phosphokinase and myokinase

When myokinase, creatine phosphokinase and the appropriate substrates are incubated together the reactions which occur are represented by equations (3) and (1). If excess of creatine phosphokinase and creatine phosphate are present the rate of creatine liberation will depend on the ADP concentration, i.e. in the coupled system on the rate of ADP formation by myokinase. It should be possible to arrange the conditions so that the rate of creatine liberation is a measure of myokinase activity, the assay of which by existing methods is by no means an easy matter.

In Fig. 7 is shown the progress curve when creatine phosphokinase, myokinase, creatine phosphate, adenylic acid and ATP are incubated together. The rate of creatine liberation is steady over a period of 10 min., but if ATP is omitted an initial lag phase appears in the progress curve. During this period the rate of creatine liberation starts very slowly and builds up until after some minutes it is equal to the rate obtained when ATP is added initially. This lag phase is presumably due to the fact that the enzyme preparations contain enough ATP or ADP to prime the autocatalytic formation of ADP by the combined action of reactions (3) and (1). Ultimately enough ATP and adenylic acid are present so that the enzyme myokinase itself rather than its substrates become limiting and the rate of creatine liberation is identical with that obtained when adenylic acid and ATP were both present in excess initially. The results illustrated in Fig. 7 were obtained with myokinase and creatine phosphokinase (Askonas, 1951) which had been dialysed with constant stirring against distilled water for 24 hr. at 0°. They indicate too why it was possible to assay adenylic acid in the absence of added ATP by the creatine phosphokinase–hexokinase–myokinase systems described earlier. In this latter system (see Fig. 6) the added adenylic acid was present in about one-hundredth of the concentration used in the experiment illustrated in Fig. 7, and hence in the presence of high myokinase and creatine phosphokinase activities and traces of ADP or ATP, adenylic acid was virtually as effective in catalysing the liberation of creatine as was the same concentration of ADP.

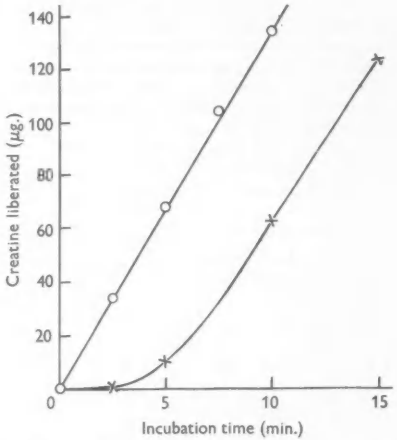

Fig. 7. Progress curve obtained with dialysed myokinase in the presence of excess of dialysed creatine phosphokinase (Askonas, 1951) at 30°. The complete system contained 1·5 μmoles adenylic acid, 0·08 μmole ATP, 10 μmoles creatine phosphate, 0·005 M-$MgSO_4$, 0·05 M Tris buffer, pH 7·4; total volume, 2·0 ml. Creatine phosphokinase was added directly before the myokinase. ○, complete system; +, without ATP.

DISCUSSION

The procedure described offers a satisfactory method of assaying creatine phosphokinase activity under the defined conditions, the only real limitation being in the choice of a buffer which does not interfere with the colorimetric determination of creatine. The method also lends itself to the study of coupled systems involving creatine phosphokinase and other enzymes which use ADP or ATP as substrate. By virtue of the fact that in these coupled systems the adenine nucleotides play coenzyme-like roles, small amounts of them will produce large overall changes in the system. The creatine phosphokinase–hexokinase system is particularly useful for the detection and estimation of small amounts of ADP and ATP. When both are present it does not distinguish between them as these substances are equally effective in catalysing creatine liberation. If the preparations are free of myokinase, ADP and ATP on the one hand can be readily distinguished from adenylic acid on the other. Usually, creatine phosphokinase prepared by the methods of Banga (1943) or Askonas (1951) are free of myokinase. The feature of this method of determination of the adenine nucleotides is its extreme sensitivity. As used in this and the following paper (Perry, 1954) the amounts of adenine nucleotide determined have been in the range 0·5–5·0 μg., but by prolonging incubation times and decreasing the final volume used for colorimetric

measurement it should be possible to increase the sensitivity by a considerable extent. The rate of creatine liberation by a particular nucleotide sample will vary with the enzyme preparations and it is necessary to determine the rate of creatine liberation with graded amounts of known standard nucleotide at the same time and under identical conditions.

The experiments indicate that even dialysed purified preparations of creatine phosphokinase are liable to be contaminated with very small amounts of ADP and ATP. It should be emphasized, however, that although these nucleotides contaminate the enzyme, the amounts must be extremely small as in the experiment shown in Fig. 6 none could be demonstrated. Here, in the absence of added ADP, using the same creatine phosphokinase as was used in the experiments illustrated in Fig. 7, there was no liberation of creatine. Nevertheless, such small amounts of ADP or ATP would be adequate to prime the autocatalytic generation of ATP from adenylic acid by the combined action of creatine phosphokinase and myokinase.

SUMMARY

1. A method for the assay of creatine phosphokinase is described. The method involves creatine estimation under conditions in which creatine phosphate is completely stable.

2. The coupled creatine phosphokinase–hexokinase system has been demonstrated to be an extremely sensitive means of determining 0·5–5·0 μg. of ADP and ATP.

3. By the addition of myokinase the creatine phosphokinase–hexokinase system can be used to determine small amounts of adenylic acid.

4. Some aspects of the coupled creatine phosphokinase–myokinase system have been studied.

During this investigation one of us (J. B. C.) was supported by a Medical Research Council Studentship. For this award and a Research Expenses Grant made by the same body (to S. V. P.), we wish to express our gratitude.

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Creatine Phosphokinase and the Enzymic and Contractile Properties of the Isolated Myofibril

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Myofibrils from rabbit skeletal muscle which have been carefully isolated and freed from other intracellular components contain a small but constant amount of nucleotide (Perry, 1952, 1953). This nucleotide consists mainly of adenosine diphosphate (ADP), and in view of the importance of the adenosine polyphosphates in cell metabolism, and in the contractile process in particular, the close association of ADP with the isolated contractile unit itself appears to be of some significance. Wherever ADP is found in the cell, enzyme mechanisms exist for its phosphorylation to adenosine triphosphate (ATP), and it is therefore reasonable to suggest, as was done in an earlier communication (Perry, 1952), that some system must be present in the muscle cell for the conversion of the myofibrillar ADP into ATP.

Such a system would have the advantage of maintaining ATP precisely localized where it is needed for contraction. At the same time, if we accept the assumption that the bound nucleotide of the myofibril is associated with the actin moiety of this structure, such an enzyme system might be involved in the transformation between F-actin (fibrous) and G-actin (globular). From the work of several investigators (Straub & Feuer, 1950; Laki, Bowen & Clark, 1950; Szent-Györgyi, 1951; Mommaerts, 1952), evidence has accumulated indicating a change in the level of phosphorylation of the nucleotide bound to actin when this protein is transformed from the globular to the fibrous form.

The present paper describes investigations made in an attempt to elucidate the mechanism of the phosphorylation of the myofibrillar nucleotide with particular reference to the role of creatine phosphokinase. The results indicate that the myofibrillar ADP does not catalyse the breakdown of creatine phosphate in the presence of creatine phosphokinase as does free ADP in the presence of the latter enzyme and myofibrils. By coupling the creatine phosphokinase system to the myofibril in the presence of extremely low concentrations of ADP, shortening is produced under conditions which strongly suggest that ATP must be split by the myofibril during this process.

A preliminary account of some of the results of this investigation has already been presented Perry (1954).

METHODS

Myofibril preparations. Myofibrils were prepared from rabbit back and hind-leg muscle by the method already described (Perry, 1953*a*). They were stored at 0° as concentrated stock suspensions in the same medium as was used for their preparation, consisting of 0·025 M-KCl and 0·039 M borate buffer pH 7·1. Even after storage for 3–4 weeks under such conditions myofibrils shortened readily with ATP or with the creatine phosphate system. Immediately before use the stock suspensions were diluted with 4–5 vol. of the KCl: borate medium, centrifuged in the cold and the supernatant discarded. This process was repeated twice and finally the suspensions were diluted with medium to give a fivefold dilution of the original stock suspension. This procedure removed most of the slight myokinase activity, but not the adenylic deaminase activity, associated with the stock suspensions of myofibrils.

Muscle acetone powder. Minced rabbit muscle was treated for 30 sec. in a Waring Blendor with 3 vol. of KCl: borate medium, pH 7·1. After being stirred for 30 min. the muscle was filtered through muslin and the residue re-extracted with 2 vol. of medium. The combined filtrates were centrifuged for 15 min. at 600 *g* to sediment myofibrils and the turbid supernatant was then precipitated with 3 vol. of acetone. Finally the precipitate was washed and dried with ether as described by Needham & Pillai (1937). The KCl: borate medium was used for the initial extraction instead of water to minimize the extraction of myofibrillar proteins (Perry, 1953).

Some acetone-powder preparations were also made from the first supernatants obtained during the preparation of myofibrils.

The acetone powder was stored at 0°, and for use 2 g. were extracted for 15 min. with 25 ml. distilled water. The extract was centrifuged and dialysed at 0° against frequently changed, stirred distilled water, for 1–2 days. Any precipitate was filtered off and discarded.

Creatine phosphokinase. Preparations were made according to Banga (1943) and Askonas (1951) as described in the previous paper (Chappell & Perry, 1954). Usually the Banga preparation, taken to the fourth stage, was concentrated by precipitation with 70% saturated $(NH_4)_2SO_4$, dissolved in a small volume of water and dialysed until free from $(NH_4)_2SO_4$.

Enzyme experiments. Experiments involving the creatine phosphokinase system were carried out essentially as described by Chappell & Perry (1954). The reaction medium, of total volume 2 ml., contained 0·05 M phosphate buffer, pH 7·4, 0·005 M-$MgCl_2$, 0·005 M creatine phosphate, 0·1–0·2 ml. acetone-powder extract or creatine phosphokinase and 0·5 ml. of washed myofibril suspension (stock diluted 1 in 5). For those experiments in which ADP was added to the system, usually 0·2 ml. of $2{\cdot}0–3{\cdot}0 \times 10^{-5}$ M ADP was used. The reactions were started by addition of either the creatine phosphokinase or the creatine phosphate. The enzyme was inactivated using phenylmercuric acetate, and deproteinization carried out by the method of Somogyi (1945) as described by Chappell & Perry (1954).

Adenosinetriphosphatase (ATPase) experiments were carried out as described earlier (Perry, 1951) using aminotrishydroxymethylmethane (Tris) buffer.

Observation of shortening of myofibrils. Samples of reaction mixture were withdrawn at frequent intervals and examined under the microscope with the aid of methylene-blue stain. Complete contraction was considered to have taken place when all the myofibrils in the field appeared granular and had clumped in irregular masses.

Creatine estimations. These were carried out as described in the preceding paper (Chappell & Perry, 1954).

RESULTS

In preliminary attempts to phosphorylate the bound nucleotide of the myofibril, creatine phosphate was incubated with a well-dialysed aqueous extract of muscle acetone powder and a suspension of repeatedly washed myofibrils. With such a system, which will be referred to as the glycolytic extract-myofibril (GEM) system, there was a rapid production of creatine. As Table 1 indicates, creatine was not produced in the absence of myofibrils or acetone-powder extract. When magnesium ion was omitted from the incubation mixture, creatine formation was usually very low and in some cases absent. Concomitant with the liberation of creatine, the myofibrils shortened and settled to the bottom of the incubation tube.

Simultaneous determinations of the creatine and phosphate liberated after incubation clearly showed that these substances were being produced in the amounts to be expected for the hydrolysis of creatine phosphate.

At an early stage in the investigation the results were explained by postulating that the bound ADP of the myofibril was converted by the creatine phosphokinase system into ATP which itself was then dephosphorylated by the myofibrillar ATPase to re-form ADP (Perry, 1954). In this manner the bound ADP could catalyse the breakdown of creatine phosphate. A similar system with hexokinase and small amounts of free ADP has already been described in the preceding paper (Chappell & Perry, 1954).

Indications that this explanation was not satisfactory were given by several observations. Creatine phosphokinase preparations purified by the method of Banga (1943) neither liberated creatine nor induced shortening when incubated with myofibrils, creatine phosphate and magnesium ions. Also, after prolonged ageing, acetone-powder extracts lost their ability to liberate creatine in the GEM system but retained their creatine phosphokinase activity. The activity of these aged acetone-powder extracts could be restored by the addition of very low concentrations of ADP (2–4 μg. per enzyme tube), and also such trace amounts of ADP similarly produced a liberation of creatine and shortening of the myofibrils when added to a reaction mixture containing purified creatine phosphokinase and myofibrils (CPM system).

This suggested that the dialysed acetone-powder extracts were active in the GEM system because they contained ADP or ATP in amounts sufficient to catalyse appreciably the breakdown of creatine phosphate. That this was the case was proved by the following experiments. When acetone-powder extracts were incubated with creatine phosphate, hexokinase, glucose and magnesium, creatine was produced only with those preparations which liberated creatine and shortened myofibrils in the GEM system. This means that these dialysed acetone-powder extracts contained enough available nucleotide to allow reactions (1) and (2) to proceed at a measurable rate (cf. Chappell & Perry, 1954).

$$\text{creatine phosphate} + \text{ADP} \rightleftharpoons \text{creatine} + \text{ATP}. \quad (1)$$

$$\text{ATP} \rightarrow \text{ADP} + \text{phosphate}. \quad (2)$$

The total amount of ADP and ATP present in various creatine phosphokinase preparations was determined using the creatine phosphokinase–hexokinase system described in the preceding paper (Chappell & Perry, 1954). Table 2 shows the results of such determinations, and it is apparent that only those preparations active in the GEM system contain significant amounts of ADP or ATP. Clearly the routine 48 hr. dialysis of acetone-powder extracts with stirring and frequent changes of distilled water, as used in these experiments, was not an effective method of removing trace amounts of ADP or ATP. In one experiment an acetone-powder extract, which was treated in this way and then dialysed for a further 12 days, still catalysed the liberation of creatine in the GEM system. The remarkable feature is that these nucleotides should survive for so long in an extract which contains some myokinase and very slight ATPase activity, and yet be readily available to the creatine phosphokinase and myofibrillar ATPase systems.

The myofibrillar ADP, however, appeared to be unavailable to the combined action of the latter two enzymes. Once removed from the myofibril by trichloroacetic acid treatment the extracted nucleotide readily catalysed the liberation of creatine by a system containing nucleotide-free creatine phosphokinase and myofibrils. For example if, as illustrated in Table 3, 1 ml. of a myofibril suspension was incubated with purified creatine phosphokinase (Banga) and creatine phosphate no creatine was liberated. When, however, half of the volume of myofibril suspension was supplemented by the nucleotide extracted from the other half, there was a considerable formation of creatine. In both cases the total nucleotide in the system, i.e. bound and free, is identical.

Table 1. *Liberation of creatine by the acetone-powder extract-myofibril system*

Myofibril preparations diluted 1 in 5. Incubations, carried out under conditions described in the Methods section, for 5 min. at 30°. Preparations *a* and *b* were 3 and 9 days old, respectively. Values quoted are μg. creatine liberated.

	Myofibril preparations	
	a	*b*
Complete system	106	109
Same without Mg^{2+}	15·2	4·3
Same without creatine phosphate	Nil	Nil
Same without myofibrils	4·5	2·0
Same without acetone-powder extract	3·0	Nil

Table 2. *Concentration of ADP + ATP in myofibril and creatine phosphokinase preparations*

Unless otherwise stated, creatine was liberated when 0·2 ml. creatine phosphokinase preparation either as the purified enzyme as an acetone-powder extract was incubated with 0·5 ml. of the 1-in-5 diluted myofibril suspension for 4 min. at 30°. ATP and ADP were extracted from the preparations by trichloroacetic acid treatment as described by Perry (1952).

	ATP + ADP (μmoles × 10^{-3}/ml.)	Creatine liberated (μg.)
Myofibrils (stock suspension)	68	—
Creatine phosphokinase	1	Nil
Acetone-powder extract	36	86
Acetone-powder extract*	69	101
Acetone-powder extract	Nil	Nil
Acetone-powder extract	2	3

* 0·6 ml. myofibrils used.

Table 3. *Bound nucleotide and the creatine phosphokinase–myofibril system*

Reaction mixture contained 0·05 M phosphate buffer, pH 7·4, 0·005 M-$MgCl_2$, 0·005 M creatine phosphate and 0·2 ml. of purified creatine phosphokinase (Banga, 1943). Total volume 2 ml., incubated for 5 min. at 30°.

Myofibril suspension added (ml.)	Nucleotide added (μmoles $\times 10^{-3}$)	Creatine liberated (μg./5 min.)
0·5	Nil	Nil
1·0	Nil	Nil
0·5	5·6 (ADP)	51
0·5	2·8 (ADP)	29
0·5	Extracted from 0·5 ml. myofibrils	37

It is also clear that the nucleotide did not leach out of the myofibrils when stored in the KCl : borate medium, for neither freshly prepared myofibrils nor the same preparations stored for several weeks and tested without further washing, liberated creatine on incubation with the purified creatine phosphokinase system (Banga). Any leaching out of nucleotide from the myofibril would be made apparent by a production of creatine in this system.

Effect of cations on the creatine phosphokinase–myofibril system

In view of the fact that the creatine phosphokinase–myofibrillar ATPase system is probably of considerable importance within the cell for the supply of energy-rich phosphate to the myofibril, this system was investigated in some detail. For the experiments described in this section, dialysed aqueous extracts of muscle acetone powder which produced creatine liberation in the GEM system, were used.

The liberation of creatine by the GEM system has already been demonstrated to be markedly dependent on the presence of Mg^{2+}. Fig. 1 shows that in the absence of this ion, the activity of the system was slight at pH 7·4, but maximal activation was obtained at quite low concentrations of Mg^{2+}, i.e. 0·002–0·003 M. Increasing the Mg^{2+} concentration up to 0·04 M produced little further effect on the amount of creatine liberated. On the other hand, with $CaCl_2$ concentrations up to 0·025 M the activity remained the same as that shown in the absence of added cations, but slight activation was obtained at higher concentrations of $CaCl_2$ (Fig. 2).

In contrast to their effects on the GEM system, Ca^{2+} and Mg^{2+} both activate the myofibrillar ATPase (Perry, 1951). Hence in the former enzyme system when Ca^{2+} alone is present the limiting feature cannot be the splitting of ATP formed, but rather the enzymic phosphorylation of ADP, a process which requires Mg^{2+}.

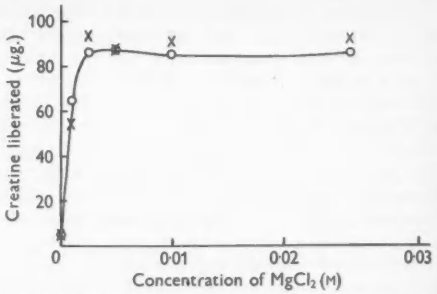

Fig. 1. Effect of Mg^{2+}, and Mg^{2+} in the presence of Ca^{2+} on the liberation of creatine by the acetone-powder extract-myofibril system. Incubations carried out in 0·05 M phosphate buffer, pH 7·4; 5 min. at 30°. ○, Mg^{2+}; ×, Mg^{2+} in presence of 0·006 M-$CaCl_2$.

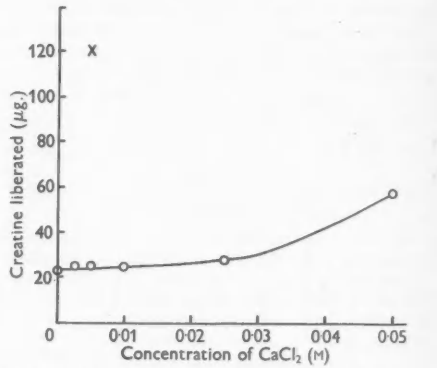

Fig. 2. Effect of Ca^{2+} on the liberation of creatine by the acetone-powder extract-myofibril system. Incubation carried out as in Fig. 1. ○, Ca^{2+}; ×, Mg^{2+} for comparison.

The dependence of the creatine liberation on the splitting of ATP by the myofibril was demonstrated with myofibrils treated so that Mg^{2+} produced little activation of the ATPase. Such preparations were obtained by treating myofibrils with M-KCl, a procedure which largely destroys the structure of the myofibril and profoundly modifies the Mg^{2+} activation of the ATPase (Perry, 1951). Table 4 presents the results of an experiment in which the rate of creatine liberation by the GEM system and the myofibrillar ATPase activity are compared before and after M-KCl treatment. The experiment was arranged so that the control and potassium chloride-treated myofibrils were compared under identical ionic conditions, and it clearly shows that simple disorganization of the myofibril as is induced by the salt treatment, diminishes in a comparable way the amount of creatine liberated and the ATP split by the respective systems.

This relation between creatine liberation and the ATPase activity of the myofibrils was further demonstrated by a comparison of the behaviour of the two systems when the ionic strength of the medium used for the enzymic tests was increased. Addition of 0·3 M-KCl decreased both the Mg^{2+}-activated ATPase and the rate of creatine liberation, although in the latter case not quite to the same extent.

When the effect of Mg^{2+} concentration on the creatine liberated by the GEM system was studied in the presence of 0·006 M-$CaCl_2$ (the value given for muscle by Dubuisson, 1951) very little modification of the curve obtained for $MgCl_2$ alone (see Fig. 1) was obtained. This suggested that these ions did not have the same antagonistic effect on myofibrillar ATPase activity such as has been demonstrated for myosin (Mommaerts & Seraidarian, 1947). A study of the effect of these two ions on myofibrillar ATPase activity confirmed this point. 0·006 M-Ca^{2+} increased the ATPase activity at low concentrations of Mg^{2+}, and when the concentration of the latter ion was 0·01 M, activities with Mg^{2+} alone and with Mg^{2+} plus 0·006 M-$CaCl_2$ were practically identical.

Under the usual test conditions the amount of creatine liberated was not affected very much by considerable variation in creatine phosphate concentration; thus, increasing the creatine phosphate concentration from 0·0005 to 0·02 M raised the amount of creatine produced in a given time by only 30 %. Increase in the amount of dialysed acetone-powder extract or in the myofibril concentration increased the rate of creatine liberation. In some cases the response to increases in the amount of dialysed acetone-powder extract was linear, but more usually it was of the type illustrated in Fig. 3.

Table 4. *Effect of treatment with* M-KCl *on creatine liberation and splitting of ATP*

Creatine was estimated after 0·5 ml. myofibril suspension and 0·1 ml. of acetone-powder extract had been incubated together with 0·05 M phosphate buffer, pH 7·4, and the usual additions. Phosphate liberated from ATP determined by simultaneous independent experiments carried out in 0·05 M Tris buffer, pH 7·3, using 0·3 ml. of myofibril suspension. In both cases 0·005 M-$MgCl_2$ and 0·1 M-KCl were present. Incubations for 4 min. at 30°.

	Creatine liberated (μg.)	Phosphate liberated (μg.)
Myofibrils treated with M-KCl	9·9	4·6
Untreated myofibrils	36·0	15·8

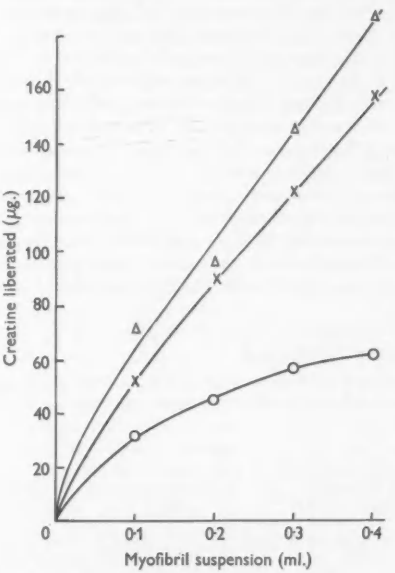

Fig. 3. Effect of variation in myofibril and acetone-powder extract concentrations on the liberation of creatine by the acetone-powder extract-myofibril system. Incubations carried out as in Fig. 1. ○, 0·1 ml.; ×, 0·2 ml.; △, 0·3 ml. acetone-powder extract.

The creatine phosphate-induced shortening of isolated myofibrils

During the course of the experiments on the splitting of creatine phosphate by the GEM system it was noticed that the myofibril suspensions flocculated and settled rapidly during incubation. More detailed study of this shortening was carried out in a medium similar to that used for the enzymic studies. This consisted of 0·05 M phosphate buffer, pH 7·4, 0·005 M-$MgCl_2$ and 0·005 M creatine phosphate (in addition to 0·006 M-KCl and 0·01 M borate buffer introduced with the myofibril suspension). When freshly prepared myofibrils and dialysed acetone-powder extract were added to this medium shortening was complete in 1–2 min. at 16°. Shortening was also readily observed with myofibril preparations which had been stored as a concentrated suspension for 3–4 weeks. Seen under the microscope the shortened myofibrils presented a very different appearance from the original structures, the characteristic myofibrillar form being absent and replaced entirely by granular bodies which had clumped together in irregular masses.

Control experiments showed that the acetone-powder extract and creatine phosphate were necessary additions for shortening to take place, but that in the absence of added Mg^{2+} slight changes could sometimes be observed after several hours. Although shortening of myofibrils was readily obtained when fresh dialysed aqueous extracts of acetone powder were used, if the latter was replaced

by purified creatine phosphokinase (Banga, 1943) no changes could be detected under the microscope. In fact it was apparent from the results of a number of experiments that in the absence of creatine liberation in the CPM system, shortening did not take place. The addition of trace amounts of ADP (2–4 μg. in 2 ml.) to systems containing purified creatine phosphokinase produced shortening and creatine was liberated. ADP was ineffective in the absence of added creatine phosphokinase or creatine phosphate, but if the ADP concentration was varied in the presence of these two substances the time taken to complete the contraction of the myofibrils could be changed from less than a minute to several hours.

An interesting feature of the system was that contraction of myofibrils could be induced within a few minutes at room temperature with low ADP concentrations, whereas the same concentration of ATP produced little change in myofibrils under conditions identical except for the absence of creatine phosphokinase. In other words, ADP which was being actively phosphorylated by the creatine phosphokinase system was much more effective than the same molar concentration of ATP. It was usually not possible to observe any significant change in myofibrils which had been treated with ATP at the same concentration as the ADP which in the presence of creatine phosphokinase produced rapid contraction. To obtain the same degree of shortening with ATP alone it was necessary to increase its concentration 10–100 times that of the ADP, suggesting very strongly that for a given low ATP concentration shortening takes place much more readily when phosphate turnover can be maintained by the creatine phosphokinase system.

In the experiments illustrated in Table 5, $5{\cdot}6 \times 10^{-3}$ μmole ATP itself was not adequate to bring about the shortening but when this concentration was maintained by supplying $5{\cdot}6 \times 10^{-3}$ μmole ADP and the creatine phosphokinase system, so that appreciable turnover of ATP resulted from the action of the myofibrillar ATPase, contraction rapidly proceeded to completion.

DISCUSSION

It is clear that for the isolated myofibril to shorten in the presence of purified creatine phosphokinase and creatine phosphate small amounts of available ADP or ATP had to be present in the system. The myofibril itself contained ADP but this did not catalyse the breakdown of creatine phosphate as did added ADP in the presence of creatine phosphokinase and myofibrillar ATPase. The possibility that the myofibrillar ADP is phosphorylated to ATP by the creatine phosphokinase system has not been excluded by the experiments carried out so far, but it is apparent that if this does take place the ATP formed is not subsequently dephosphorylated by the myofibrillar ATPase.

The observation that aqueous extracts of muscle powder contain small amounts of ADP or ATP which are not readily removed by conventional means, raises the question as to whether the small quantities of nucleotides associated with other components from muscle, e.g. myofibrils and actin, are simply present as contaminants with no functional significance. Such an association might be produced by the formation of polyvalent metal complexes with the nucleotide and the protein (cf. Buchthal, Deutsch, Knappeis & Munch-Petersen, 1951). In the case of actin, with which the bound nucleotide of the myofibril is presumably associated, the evidence of changes in nucleotide composition during the G-actin–F-actin transformation (Straub & Feuer, 1950; Laki *et al.* 1950; Szent-Györgyi, 1951; Mommaerts, 1952) certainly suggests a functional significance. The present investigation shows that the ADP is specifically bound to the myofibril and is not available to the creatine phosphokinase–myofibrillar ATPase system, in clear

Table 5. *Shortening of isolated myofibrils by ADP in the presence of the creatine phosphokinase system and by ATP*

All tubes contained 0·05 M phosphate buffer, pH 7·4, 0·005 M-$MgCl_2$, 0·005 M creatine phosphate and 0·5 ml. myofibril suspension. ADP and 0·2 ml. of nucleotide-free creatine phosphokinase was added to one series and ATP alone to the other. Total volume 2 ml.

	ADP + creatine phosphokinase		ATP alone		
Serial no.	ADP present (μmoles $\times 10^{-3}$)	Contraction time (min.)	ATP present (μmoles $\times 10^{-3}$)	Contraction time (min.)	Temp. (°)
13/6/53	5·6	15 (completely granular)	5·6	50 (no obvious change)	—
			56	48 (contraction obvious but not complete)	18
18/6/53	5·6	20 (completely granular)	5·6	100 (little change)	—
			56	100 (obviously shortened but not completely granular)	18
26/6/53	11·2	8 (completely granular)	11·2	240 (very slight contraction)	—
			112	12 (completely granular)	23

distinction to the nucleotide found in the acetone-powder extracts.

Since this work was completed, Goodall & Szent-Györgyi (1953) have published observations on the role of creatine phosphate and a factor *X* isolated from aqueous muscle extract, in the relaxation of glycerated fibres. Factor *X* is precipitated between 50 and 70% saturation with ammonium sulphate and must contain creatine phosphokinase, for the latter enzyme precipitates in this range. The enzymic system employed by Goodall & Szent-Györgyi is very similar to that reported here and contains in addition to factor *X*, creatine phosphate, ATP and Mg^{2+}. Under these conditions the ATP level will be maintained in the same way as has been described for the myofibril systems, despite continuous breakdown by the glycerated fibre. Relaxation was measured by the fall in tension when the pH value of the medium containing the isometric fibre was lowered to 5·7. A possible explanation is that relaxation takes place under these conditions simply due to the inhibition of the fibre ATPase by the low pH. Under these conditions little splitting of ATP can take place, the fibre shows little tendency to shorten, and tension is not maintained, although the ATP level is kept constant by the creatine phosphokinase system. It is significant that a similar effect is reported by these authors with ATP alone, although as one might expect the relaxation is much less than that obtained when factor *X* and creatine phosphate are present, for under the conditions of assay the latter system is much more effective in maintaining the ATP level.

The system described involving myofibrils, creatine phosphokinase, ADP and creatine phosphate offers a means of studying both physical change and enzyme activity of the myofibril in the presence of a constant concentration of ATP. Furthermore, by decreasing the ADP concentration the rate of contraction can be made correspondingly slower, and at the same time the rate of splitting of creatine phosphate falls in a similar manner (cf. creatine phosphokinase and hexokinase system, Chappell & Perry, 1954). These facts, like the evidence already produced by Weber & Portzehl (1952), support the view that the splitting of ATP induces contraction. Other investigators, e.g. Szent-Györgyi (1953) and Hill & Morales (1952), maintain that for contraction to take place only the adsorption of ATP by the contractile system is necessary, the splitting of ATP taking place during the relaxation process. This latter suggestion is extremely difficult to disprove experimentally, but the above facts together with the observation that a given low concentration of ATP, maintained by the creatine phosphokinase system, is much more effective for inducing contraction than the same concentration of ATP alone, indicates that the splitting of phosphate from ATP is very closely associated with the shortening of the isolated myofibril. If adsorption alone is sufficient to induce contraction it is difficult to see why a given ATP concentration should be less effective in bringing about contraction of the myofibril than a much lower concentration of ATP maintained by the creatine phosphokinase system.

SUMMARY

1. At pH 7·4 in the presence of well-dialysed aqueous extracts of muscle acetone-powder, creatine phosphate and magnesium ions, isolated myofibrils shorten and simultaneously creatine phosphate is split.

2. This shortening process and splitting of creatine phosphate has been demonstrated to be due to low concentrations of ADP or ATP which are associated with the acetone-powder extracts and not readily removed by dialysis.

3. The bound ADP of the myofibril does not catalyse creatine phosphate breakdown in the creatine phosphokinase-myofibril system.

4. The effects of calcium and magnesium ions on the liberation of creatine by the acetone-powder extract-myofibril system have been described.

5. In the presence of the creatine phosphokinase system, low concentrations of ADP will bring about rapid contraction of isolated myofibrils, whereas a similar concentration of ATP added in the absence of creatine phosphokinase induces little or no change. This observation is taken as evidence that removal of phosphate from ATP is essential for contraction of the isolated myofibril.

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Studies in Carotenogenesis

12. THE EFFECT OF TEMPERATURE AND THIAMINE CONCENTRATION ON CAROTENOGENESIS BY *PHYCOMYCES BLAKESLEEANUS*

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In *Rhodotorula glutinis*, variations in the temperature of cultivation have a profound qualitative effect on carotenogenesis (Skoda, 1951; Nakayama quoted by Mackinney, 1952). Low-temperature cultivation (5°) results in the production of yellow cultures containing predominantly α- and β-carotene, whilst at higher temperature (25°) the cultures become red owing to the production of the more unsaturated xanthophylls, torulin and torularhodin. In *R. sanniei*, however, temperature changes between 14 and 28° have no qualitative effect on pigmentation (Fromageot & Tschang, 1938).

Turian (1953) has recently also observed a temperature effect on carotenogenesis by *Mycobacterium phlei*. At 30° leprotene, γ-carotene, a 'rhodopin-like' carotene, a hydroxycarotene and traces of phytofluene are produced together with very small amounts of the acidic chrysophlein. At 37° only small amounts of these neutral compounds were formed, but the synthesis of chrysophlein was considerably increased.

Starr & Saperstein (1953) observed that *Corynebacterium poinsettiae* cultured in a medium low in thiamine (0·1 μg./100 ml.) is pink and contains mostly spirilloxanthin and lycoxanthin. On a high-thiamine medium (100 μg./100 ml.) orange colonies are formed which contain much less spirilloxanthin, the same amount of lycoxanthin and some cryptoxanthin, which was not present in the organisms grown in the low-thiamine medium.

The present paper describes an investigation undertaken to find out the effects of variation in both the temperature of cultivation and in the thiamine content of the medium on carotenogenesis in *Phycomyces blakesleeanus*.

EXPERIMENTAL

Cultures. Phycomyces blakesleeanus (−strain) was grown in 8 oz. medicine bottles in 15 ml. of our standard medium. This medium is that described by Schopfer & Jung (1935), but contains 2·5% instead of 10% (w/v) glucose; this concentration was found to give maximal β-carotene production (Garton, Goodwin & Lijinsky, 1951). In the experiments on temperature variation, two glass incubators (Garton *et al.* 1951) were placed close together in the laboratory, one being kept at 25° and the other at either 20 or 30°. Thus both the control (25°) and the experimental runs were always carried out under comparable conditions of illumination. The experiments at 3–5° were carried out in the dark in a 'warm' corner of a large refrigerator. It was not possible to illuminate the cultures in these experiments.

In the experiments on thiamine-deficient media, inoculation was not carried out by the usual means of a spore suspension made by shaking up a stock agar culture of *Phycomyces* with sterile water (Garton *et al.* 1951), but by a very small direct transfer with a needle from an agar slope, care being taken not to transfer any agar. This precaution was necessary to eliminate the possibility of carrying over significant amounts of thiamine. That this was achieved, was shown by the fact that no growth (or occasionally very little) occurred on media containing no thiamine.

On repeating the experiments on thiamine-low media some time after the first series was carried out, it was found that no growth could be obtained. This failure was eventually traced to the age of the parent culture. Inoculations from cultures up to 6 months old will grow quite well on the standard medium, but in order to get growth in thiamine-low media, young parent cultures (less than 4 weeks old) must be used.

Determinations of dry weight and carotene. Dry weight and carotene determinations were carried out according to the methods described by Garton *et al.* (1951). The chromatographic separation of the carotenoids was carried out according to the method of Goodwin (1952).

In the experiments on the low-thiamine media the dry-weight production per medicine bottle was often very small (approx. 5 mg.). In order to reduce the error in the dry-weight and β-carotene determinations at this low level, mycelia from five to six bottles were bulked for each determination.

RESULTS

Effect of temperature on growth, lipogenesis and carotenogenesis. Two experiments were carried out at 20, 25 and 30° and the results of one are given in Table 1. Each figure is the result of two determinations on the combined mycelia of two bottles. It will be seen that the temperature of cultivation has no obvious effect on production measured as dry weight, that lipogenesis decreases somewhat with increasing temperature and that β-carotene synthesis was greatest at 25°, being less at both 20 and 30°. The possibility existed that the 30° cultures had passed their maximal dry weight production before 4 days. This was considered unlikely, the value for 4 days (101 mg.) at 30° being probably rather exceptional. To check this, however, two further experiments were carried out at 30° to determine whether maximal dry-weight production occurred before 4 days. The results were very similar in both experiments, and the mean values are recorded in Table 2. It will be seen that in this experiment the dry-weight production was rather less than in the experiments recorded in Table 1. Maximal dry-weight production occurred 6 days after inoculation, growth was by no means complete on the fourth day and insufficient mycelium was produced on the third day to warrant examination. It will be seen that compared with results in Table 1, lipid and carotene synthesis is still somewhat lower at 30° than at 25°. A direct comparison could not, however, be made because no controls at 25° were run, as the main interest was in the time at which maximal dry-weight production occurred. At 3–5° growth was very slow and sufficient amounts of mycelia for examination were produced only 20 days after inoculation, even then growth was not complete (see Table 3). It did however appear to be complete after 26 days. It will be seen that very

Table 1. *Growth, carotene and lipid production by* Phycomyces *cultured at different temperatures*

Standard medium: all quantities expressed as amounts produced on 15 ml. medium in 8 oz. medicine bottles.

	20°				25°				30°			
Age of culture (days)	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)	Total dry wt. (mg.)	Lipid (mg.)	Lipid concn. (%)	Carotene concn. (mg./100 g. dry wt.)
4	111	34·7	31·3	13·2	109	35·0	32·4	37·3	101	24·7	24·5	60·3
6	119	35·0	29·4	39·2	114	29·0	25·4	59·8	88	20·9	23·8	55·4
8	114	34·1	29·9	66·0	106	25·1	23·7	113·1	91	18·2	20·0	87·8
10	101	26·7	26·4	82·3	103	21·8	21·2	129·0	96	17·1	17·8	79·9

Table 2. *Growth, β-carotene and lipid production by* Phycomyces blakesleeanus *cultured at* 30°

Standard medium: all quantities expressed as amounts produced in 15 ml. medium in 8 oz. medicine bottles.

Age of culture (days)	Dry wt. mycelium (mg.)	Lipid (mg.)	Lipid concn. (%)	β-Carotene (μg.)	β-Carotene concn. (mg./100 g. dry wt.)
3	Insufficient growth for analysis				
4	38·8	10·4	26·8	15	38·7
5	60·6	15·6	25·8	35	57·8
6	76·8	14·9	19·4	54	70·3
7	78·3	14·7	18·8	56	71·5

Table 3. *Dry weight, lipid and β-carotene synthesis in* Phycomyces blakesleeanus *grown at 3–5° in the dark*

15 ml. of standard medium in 8 oz. medicine bottles, each figure represents the mean of 4 determinations.

Age of culture (days)	Dry wt. mycelium (mg.)	Lipid (mg.)	Lipid concn. (%)	β-Carotene (μg.)	β-Carotene concn. (mg./100 g. dry wt.)
20	61·4	14·2	23·2	14	22·8
26	91·0	21·8	24·0	13	14·3
Control* (mean 8–13 days in dark at 25°)	88·0	15·8	18·0	76	86·4

* From Garton *et al.* (1951).

Table 4. *Growth and carotene synthesis by* Phycomyces blakesleeanus *on media low in thiamine*

Standard medium containing varying amounts of thiamine: 6-day cultures; temp. 25°. All quantities expressed as amounts produced by 15 ml. medium in 8 oz. medicine bottles; five to six cultures bulked and analysed at each concentration of thiamine.

Thiamine concn. (mg./100 ml.)	Dry wt. mycelium (mg.)		β-Carotene (μg.)		β-Carotene concn. (mg./100 g. dry wt.)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0·02	5·2	2·1	1·7	0·5	32·3	24·6
0·04	4·7	—	1·3	1·9	27·5	—
0·08	9·4	6·9	6·4	4·1	67·5	60·4
0·10	8·2	7·8	4·8	4·9	58·0	62·7
0·12	11·7	9·7	8·9	8·9	78·5	91·6
0·16	13·1	11·6	10·4	12·1	79·0	104·9
0·20	12·9	10·3	11·1	9·6	86·0	92·6
Control (25)	—	91·7	—	85·0	—	92·6

little β-carotene is produced at this low temperature, although the dry weight of the mycelium eventually reaches a normal value. Lipid synthesis is a little higher than observed earlier in the dark at 25° by Garton *et al.* (1951).

Chromatography of the total carotene fractions produced at different temperatures showed that all were quite normal in pigment distribution and that even at low temperatures, when β-carotene synthesis was considerably reduced, there was no corresponding increase in the synthesis of unsaturated polyenes such as phytoene and phytofluene.

Effect of thiamine on carotenogenesis. It is well known that, at concentrations of thiamine less than 2·0 μg./100 ml. of medium, the growth of *Phycomyces* is proportional to the amount of thiamine (see Schopfer, 1942). Preliminary experiments indicated that the concentration of carotene in the mycelium was reduced when *Phycomyces* was cultured on media containing less than 0·6 μg. thiamine/100 ml.

The effect of these low levels of thiamine on the production of carotene was investigated in greater detail. The usual 6-day cultures were used in these experiments because Schopfer (1942) has shown that, although the final growth is reduced on low-thiamine media, the rate of growth is little affected. In order to minimize errors, the 6-day cultures from five to six bottles were bulked for the dry-weight determination and the β-carotene analysis. This meant that the total dry weight measured was at least 10 mg. and the β-carotene 5–10 μg.

The results of two experiments are given in Table 4. It will be seen that in confirmation of the preliminary experiments at very low concentrations of thiamine, there does appear to be an inhibition of the synthesis of β-carotene, although the carotene production appears to reach normal levels at slightly lower thiamine concentrations (0·12–0·16 μg./100 ml.) than was first indicated (0·6 μg./100 ml.).

Chromatography of these extracts did not reveal any differences from normal extracts; in particular there did not appear to be any increase in the production of unsaturated polyenes.

It is also obvious from Table 4 that, on thiamine-low media, the carotene concentration is normal long before growth reaches its maximal value. As stated previously, this was found to occur at a thiamine level of 2·0 μg./100 ml.

DISCUSSION

The effect of temperature variations on carotenogenesis by *P. blakesleeanus* is quantitative only, and no differences in the types of polyenes synthesized were noted over the range 5–30°. There is, however, an optimum temperature for carotene synthesis at about 25°. The qualitative observations are similar to those of Fromageot & Tschang (1938) who found no changes in the pigments produced by *Rhodotorula sannei* over the temperature range 14–28°. In the closely related *R. glutinis*, however, the pigments produced at low temperatures (5°) were different from those produced at normal temperatures (Skoda, 1951; Mackinney, 1952). There are no reports of quantitative experiments on *Rhodotorula* spp.

P. blakesleeanus in its reaction to variations of temperature, also differs from *Myco. phlei* in which a lowering of the incubation temperature from 37 to 30° considerably affects the relative amounts of the various carotenoids produced (Turian, 1953).

As in the case of the experiments in which the cultivation temperature was altered, it was found that quantitative effects on carotenogenesis could be obtained by lowering the thiamine levels of the medium. This occurred only at the lowest levels examined, 0·02–0·16 μg. thiamine/100 ml. There was no indication, however, of any qualitative changes similar to these observed in *C. poinsettiae* cultured on media containing different levels of thiamine (Starr & Saperstein, 1953).

It is interesting that in the low-thiamine media pyruvate accumulates in considerable amounts (Griffiths, 1953). Whether this means that pyruvate is an intermediate in the formation of β-carotene is not known, but when tested under other conditions pyruvate did not stimulate pigment synthesis (Goodwin, Lijinsky & Willmer, 1953).

SUMMARY

1. β-Carotene synthesis by *Phycomyces blakesleeanus* was greater at 25° than at 3–5, 20 or 30°. At all temperatures, β-carotene was the major pigment produced. There was no indication of the synthesis of enhanced amounts of more saturated polyenes at temperatures where β-carotene synthesis was reduced.

2. β-Carotene synthesis is inhibited in *Phycomyces blakesleeanus* grown on media containing less than 0·2 μg. thiamine/100 ml., whilst growth inhibition begins below a much higher level (2·0 μg./100 ml.). No qualitative changes in polyene synthesis were associated with this inhibition of carotenogenesis.

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Chemical Analysis of Human Foetal Skull Bones

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Pressures exerted on the foetal head during labour cause it to alter its shape, and this ability of the head to 'mould' in response to external pressure becomes less marked as the foetus ages. The present work was an attempt to correlate changes in chemical composition of the bones with the increasing 'hardness' of the maturing foetal head.

There have been several investigations into the chemical composition of foetal bones, but the only reports referring to the cranial bones are those of Yoshida (1930) and Toverud & Toverud (1933). Yoshida analysed the parietal bone for calcium and phosphorus, and related his findings to the length of gestation. These showed that whereas the absolute amounts of phosphorus and calcium in this bone increased as pregnancy advanced, the percentage of phosphorus decreased while that of calcium increased. This finding was surprising as was the fact that at a foetal age of 10 months the Ca/P ratio of the parietal bones was over four.

Toverud & Toverud (1933) analysed a small portion (2 × 1·5 cm.) of the parietal bone for the percentage calcium per dry weight of the sample, and the percentage calcium and phosphorus of ash. The findings were related to the length of gestation of the foetus, and no obvious relationship between percentage of calcium and foetal age was noted. The authors, however, found a sudden increase in percentage of calcium in the section of parietal bone of full-term babies as compared with the values found for the premature infants up to and including 38 weeks.

METHODS

Material

The bones for this study were obtained from still-births or from infants who had died very soon after birth. The age of the foetus was estimated from the menstrual history of the mother, only singletons were used and any foetus which showed obvious signs of possible skeletal deformity was discarded.

The bones were removed *post mortem* and kept in 0·9% (w/v) NaCl in a refrigerator until they were prepared for examination, which was always within 5 days of death. The parietal and frontal bones only were used. The medial border of each parietal bone was not included in the specimen, nor was the orbital part of the frontal bones included. Before the bones were examined the soft tissues on each surface of the bone were removed by stripping.

Chemical analysis

The concentration of calcium, phosphorus, carbonate and collagen present in the bones was determined, the results being expressed as % dry wt. The following procedure was adopted to prepare the bones for analysis. Each bone was cut into pieces approximately 2 × 1 cm. and the pieces from one bone were placed in a small beaker which was surrounded by CO_2 snow. When the pieces were hard they were pulverized to a fine powder. The powder was then placed in an H_2SO_4 desiccator, where it remained for a week or longer before being transferred to an oven whose temperature was 100°; there was no evidence that the H_2SO_4 in the desiccator affected the carbonate content of the bone powder. After at least 6 hr. in the oven it was found that further heating did not reduce the weight of the samples.

The dry, powdered bone was then ready for the estimation of carbonate. To determine the concentrations of calcium, phosphorus and collagen, approximately 0·2 g. of the dry, powdered bone was accurately weighed into an ampoule. Exactly 10 ml. of 6N-HCl were added to the ampoule, which was then sealed. The contents of the ampoule were hydrolysed either by heating for 3 hr. in a pressure cooker at 60 lb./sq.in. or left overnight in a boiling water bath. After heating, the ampoule was opened and the contents were filtered. An ampoule to which no bone powder had been added was used as a control.

The filtrate or bone solution (1 ml.) was pipetted into a 50 ml. volumetric flask followed by 1 ml. of 6N-NaOH and 2 ml. of 8% trichloroacetic acid. The solution was made up to the 50 ml. mark with distilled water and then shaken. The addition of the trichloroacetic acid never caused any precipitation, so further filtering was, in every case, unnecessary.

Estimation of calcium. The estimation of the calcium was based on the method described by Kramer & Howland (1926) in which the calcium was precipitated as the oxalate, the precipitate then dissolved in acid and the solution titrated while hot with potassium permanganate. The error of the method was ±0·5%.

Estimation of phosphorus. The method of Fiske & Subbarow (1925), with modifications by King (1932), was employed to determine the phosphorus content of the bone solution. The error of the method was ±1%.

Estimation of carbonate. Dry, powdered bone was used, and the method was that of Van Slyke (1917) with the modification suggested by Bowes & Murray (1935). The error of the method was ±5%.

Estimation of collagen. The method of Neumann & Logan (1950*a*, *b*) for the estimation of hydroxyproline was used to determine the collagen concentration of the bone solution. The error of the method was ±5%.

RESULTS

Table 1 shows the mean percentages of dry weight of calcium, phosphorus, carbonate and collagen for each of the twenty-one cases examined. The figures for each substance, in the majority of cases, are the mean values of twelve estimations, three on each of the two frontal and three on each of the two parietal bones. Also shown in Table 1 is the estimated time of gestation.

It will be seen that as pregnancy advances there is an overall increase in the percentage of calcium and carbonate in the foetal bones while the proportions of phosphorus and collagen are unaltered by foetal age, in the period under investigation.

Evaluation of results. There is a statistically significant increase in the percentage of calcium

Table 1. *The mean percentage (dry weight) of calcium, phosphorus, carbonate and collagen in foetal skull bones against the age of the foetus*

Each value is calculated from the mean of triplicate estimations of the parietal and frontal bones of an individual foetus. The figures within brackets are the standard error of the mean. Student's *t* test was applied in order to find the *P* values.

Foetus	Age (weeks)	Calcium (%)	Phosphorus (%)	Carbonate (%)	Collagen (%)
A	28	23·50 (±0·17)	10·68 (±0·10)	3·63 (±0·16)	28·4 (±0·33)
B	29	24·31 (±0·12)	11·41 (±0·06)	3·80 (±0·15)	28·2 (±0·13)
C	29	24·43 (±0·32)	10·78 (±0·14)	4·03 (±0·11)	29·1 (±0·90)
D	32	24·27 (±0·34)	11·00 (±0·08)	4·08 (±0·10)	28·3 (±0·40)
E	32	24·01 (±0·21)	10·95 (±0·05)	4·19 (±0·05)	27·6 (±0·13)
F	32	24·40 (±0·08)	10·79 (±0·10)	4·17 (±0·05)	28·3 (±0·45)
G	33	24·23 (±0·27)	10·81 (±0·07)	4·50 (±0·04)	27·3 (±0·30)
H	36	24·66 (±0·05)	10·67 (±0·13)	4·71 (±0·02)	27·8 (±0·75)
I	36	23·85 (±0·15)	10·65 (±0·06)	4·01 (±0·09)	27·8 (±0·30)
J	36	24·46 (±0·12)	10·49 (±0·02)	4·60 (±0·02)	27·9 (±0·24)
K	37	24·73 (±0·07)	10·71 (±0·10)	4·54 (±0·10)	28·5 (±0·47)
L	38	24·17 (±0·20)	10·76 (±0·04)	4·52 (±0·04)	28·6 (±0·15)
M	38	24·53 (±0·14)	11·29 (±0·03)	4·20 (±0·04)	26·6 (±0·33)
N	40	24·37 (±0·10)	10·79 (±0·03)	4·84 (±0·04)	28·7 (±0·24)
O	40	24·73 (±0·22)	10·63 (±0·15)	4·65 (±0·06)	27·5 (±0·22)
P	41	24·60 (±0·20)	11·15 (±0·07)	4·24 (±0·08)	27·8 (±0·95)
Q	42	24·28 (±0·16)	10·50 (±0·06)	4·47 (±0·03)	29·0 (±0·42)
R	42	24·10 (±0·08)	10·79 (±0·06)	4·54 (±0·07)	29·0 (±0·33)
S	43	24·81 (±0·04)	10·86 (±0·03)	4·81 (±0·07)	27·1 (±0·56)
T	43	24·85 (±0·11)	10·81 (±0·05)	4·46 (±0·10)	29·8 (±0·38)
U	43	24·63 (±0·13)	10·64 (±0·04)	4·79 (±0·05)	27·4 (±0·43)
P	—	0·02–0·01	0·3–0·2	0·001	0·9

($x = 23{\cdot}06 + 0{\cdot}037y$) and of carbonate ($x = 2{\cdot}46 + 0{\cdot}052y$) with foetal age, whereas the percentage of phosphorus ($x = 11{\cdot}24 - 0{\cdot}012y$) and collagen ($x = 28{\cdot}35 - 0{\cdot}005y$) show no such correlation where x = foetal age and y = percentage of the material investigated.

Differences in the rate of change of calcium, phosphorus and carbonate between the left and right parietal and left and right frontal bones were tested by comparing the slopes of regression lines. The differences found were not significant. Similarly, any differences between frontal and parietal bones were shown to be not significant.

DISCUSSION

As the foetus becomes mature there is an increase in the total amount of calcium, phosphorus, carbonate and collagen in those bones of the cranial vault examined, because there is no decrease in the proportion of these substances while the size of the head is increasing as is also the thickness of the parietal bone (Macdonald, 1953, 1954). During the period of pregnancy under review the chemical composition of the growing parietal and frontal bones must be changing, because the approach of maturity is associated with an increased percentage of calcium and carbonate while the percentage of phosphorus is unaltered. The chemical changes found in the bones of the cranial vault of the foetus are changes taking place in membrane bone, which has an entirely different method of development from the usual cartilage bone. It is of interest to see to what extent the theories of the inorganic structure of cartilage bone can be applied to the results described in this paper.

Bassett's (1917) views of bone as a mixture of apatite and calcium carbonate cannot be applied to these foetal bones, as the Ca/P ratio for non-carbonate Ca is too low for apatite. In like fashion the views of Kramer, Yuska & Steiner (1939) on the effect of age on bone do not apply. Bowes & Murray (1935) made three alternative suggestions to explain the distribution of calcium in dentine, the Ca/P ratio of which is very similar to the mean residual Ca/P ratio of foetal skull bones. Of these three possibilities the one that might be applied suggests the presence of calcium phosphate ($Ca_3(PO_4)_2$) as a constituent.

It is probable that increasing amounts of calcium carbonate are laid down in the bones of the cranial vault as the foetus matures, and therefore any increased 'hardness' that is found in these bones may be, in part, due to this greater proportion of calcium carbonate present at maturity. The proportion of collagen in the bones examined did not appear to have any relation to the age of the foetus in the period under review and as the organic matrix of bones consists of 90–96 % collagen or collagen-like proteins (Rogers, Weidmann & Parkinson, 1952) there does not appear to be any very marked change in the organic composition of the parietal or frontal bones of the foetus in the last 3 months of pregnancy.

SUMMARY

1. Chemical analysis of the frontal and parietal bones of the human foetus has shown an increase in the proportions of calcium and carbonate as the foetus approaches maturity, but no such increase in phosphorus and collagen. The mean proportional increase per dry weight of bone per week is 0·04 % for calcium and 0·05 % for carbonate.

2. The inorganic chemical composition of growing membrane bone, as exemplified by the foetal parietal and frontal bones, may consist of a mixture of apatite, brushite and calcium carbonate or of a mixture of calcium phosphate, calcium carbonate and small amounts of other calcium salts. It is impossible, from the results obtained here, to indicate which is the more likely chemical arrangement.

3. A probable increase in the proportion of calcium carbonate in the skull bones as the foetus grows may be a factor in the increased 'hardness' of the head associated with foetal maturity.

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The Use of Paper Partition Chromatography for Taxonomic Studies of Land Snails

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Buzzati-Traverso & Rechnitzer (1953) have shown that comparisons of the paper chromatograms obtained from tissue extracts of fishes indicate differences between species, and that within a species the patterns are remarkably constant irrespective of the size or age of the fish. They claim also that comparable results have been obtained in other phyla, and that preliminary results suggest that the technique can be used to distinguish stocks of the same species belonging to geographically isolated populations. In another study Buzzati-Traverso (1953) has found also that paper chromatography of tissues of genetically known strains of *Drosophila melanogaster* and of certain plants gives a constant and distinctive pattern for each strain. This work suggests that chromatographic analysis of tissue extracts may be of value not only as an important tool in taxonomic and population genetics studies, but, as Buzatti-Traverso points out, as an aid to understanding the biochemical basis of individuality.

In order to test the validity of Buzzati-Traverso's procedure when applied to an invertebrate phylum, a rapid method of analysis has been developed based on the use of chromatography on filter-paper disks (Rutter, 1950; Giri, 1953). Several species of land snail were used as experimental material, and the resultant patterns have been studied to determine the effect of size of animal and of variable factors in both natural and artificial environments. The results indicate that the method is likely to be of great value in the taxonomic study of land snails.

EXPERIMENTAL

Methods

Paper chromatography was carried out using circles of Whatman no. 3 paper diameter 12·5 or 15·0 cm. Extracts of tissue cut from living specimens were made by crushing small pieces of the tissue 1·5 cm. from the centre of the paper, the amount of tissue being such that when compressed and rotated with moderate manual pressure using a small glass stopper a spot approximately 0·5–0·75 cm. diameter was produced. Generally four spots were prepared on each circle. The spots were allowed to dry in a current of warm air. If kept dry the prepared papers could be stored for several days before chromatographing without noticeable effects on the resultant patterns. The solvent finally adopted was butanol:acetic acid:water (100:22:50, v/v). The solvent was contained in a 10 cm. diameter Petri dish, the paper being placed on a glass plate on top of the Petri dish and covered with an inverted pie dish. A filter-paper wick descending through a hole in the plate fed solvent to the centre of the filter-paper disk. All chromatography was carried out in a constant temperature room at $20 \pm 1°$ for 4–6 hr. At the end of the run the disk was dried horizontally in a current of warm air and examined for the ultraviolet fluorescence and absorption pattern using a Wood's glass mercury-vapour discharge lamp. R_F values of the visible fluorescent and absorption bands were measured directly with calipers whilst the pattern was being examined under the mercury-vapour discharge lamp. Photographic records of the pattern were made by printing through the paper disk. Full details of the fluorescent pattern are not reproducible photographically, however, since the filter paper itself fluoresces faintly and obscures coloured bands of low intensity which are clearly visible to the eye.

Materials

The patterns from seven species of land snails are described below. The species were: *Theba pisana* (Müll), *Helix aspersa* (Müll), *Bothriembryon indutus* (Menke), *B. kingii* (Gray), *B. dux* (Pfeifer), *B. leeuwinensis* (Smith), *Austrosuccinia contenta* (Iredale).

The first two species are common European snails first reported in Australia about 60 years ago. The species of the genus *Bothriembryon* and *A. contenta* are native to West Australia.

Specimens of *T. pisana* were collected from four different localities, namely beach dunes at Cottesloe, Albany and Hopetoun and along the river foreshore in Perth. *H. aspersa* was obtained from house gardens in Perth. All the specimens of native snails were topotypes of species listed by Iredale (1939).

The tissue used for preparing extracts was cut from the posterior or lateral edges of the foot. If the animal was withdrawn into the shell or had an epiphragm it was placed in water until the foot was extended. The shells were numbered to facilitate comparisons of the chromatographic patterns from individuals within a species.

In one experiment young specimens of *T. pisana* collected in the field were reared for 4 months in the laboratory on different diets. The animals were kept in glass jars and divided into three groups. Group 1 was fed on lettuce leaves, group 2 on buffalo grass (*Stenotathrum secundatum*) and group 3 was provided with a diet of filter paper impregnated with a mixture of yeast, cod liver oil and calcium carbonate (cf. Wagge, 1952). The food was changed twice weekly.

RESULTS

Differences between species

Each of the species of snail studied gave characteristic chromatographic patterns which could be distinguished clearly by their ultraviolet absorption and fluorescence patterns. The main characteristics of the various patterns are recorded in Fig. 1. No attempt has been made as yet to identify chemically the various components.

Effect of age and geographical locality

Three different age groups of *H. aspersa* and *T. pisana* were collected in the field. Specimens of either species were feeding on a single plant species in a restricted area. No significant differences between young, intermediate or mature animals could be detected in the characteristic patterns for either species, even when the R_F values for the four principal fluorescent bands in the pattern of *H. aspersa* and the R_F value of the outer edge of the fluorescing zone in the pattern of *T. pisana* were measured for each specimen (Table 1).

No significant difference was found between mature specimens of *T. pisana* from four widely separated localities (Table 2).

Effect of different diets on Theba pisana

A large number of very young specimens of *T. pisana* were collected on the beach dunes at Cottesloe and were reared in glass jars on the three different diets described in the previous section.

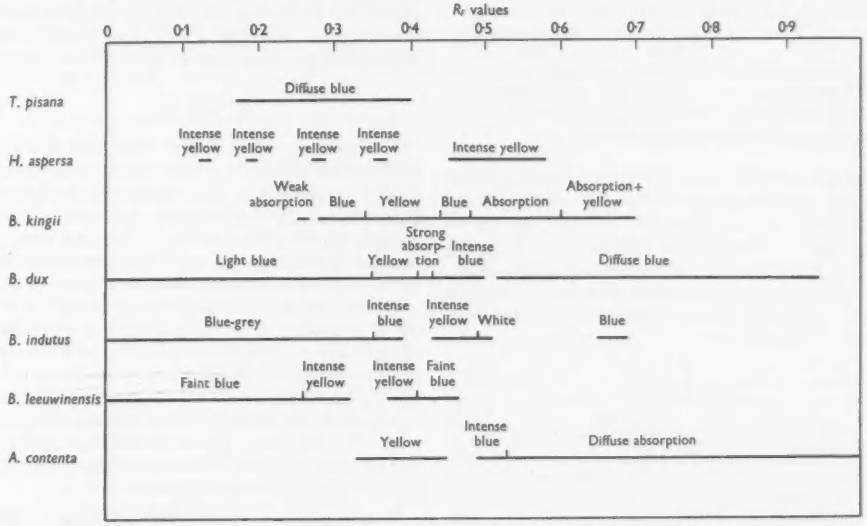

Fig. 1. Characteristic fluorescence and absorption patterns obtained by chromatographing fresh tissue squashes of the foot muscle of various land snails.

Table 1. *Mean R_F values for the main characteristics in the chromatographic pattern of fresh tissue extracts of snails of different ages*

A. *Helix aspersa* (12 animals/group)

Age group	R_F values for four principal fluorescent lines (mean ± S.E.)			
	1	2	3	4
Young	0·16 ± 0·025	0·26 ± 0·028	0·35 ± 0·028	0·51 ± 0·019
Intermediate	0·17 ± 0·018	0·28 ± 0·015	0·37 ± 0·024	0·55 ± 0·014
Mature	0·18 ± 0·032	0·28 ± 0·029	0·36 ± 0·035	0·54 ± 0·030

B. *Theba pisana* (16 animals/group)

Age group	R_F values for edge of fluorescent flare	
	Mean ± S.E.	Range
Young	0·43 ± 0·007	0·40–0·50
Intermediate	0·41 ± 0·009	0·34–0·42
Mature	0·39 ± 0·007	0·33–0·42

Table 2. *Mean R_F values from the chromatographic pattern of fresh tissue extracts of mature specimens of* Theba pisana *from different geographical localities*

Locality	No. of animals	R_F values for edge of fluorescent flare: Mean ± S.E.	Range
Cottesloe	16	0·39 ± 0·007	0·33–0·43
Perth	15	0·38 ± 0·010	0·35–0·39
Albany	16	0·43 ± 0·007	0·38–0·46
Hopetoun	16	0·39 ± 0·005	0·35–0·41

Table 3. *Mean R_F values from the chromatographic pattern of fresh tissue extracts of* Theba pisana *reared in the laboratory on different diets*

Four animals per group.

Diet	R_F: Mean	Range
Buffalo grass	0·38	0·34–0·42
Lettuce	0·37	0·35–0·40
Synthetic	0·35	0·33–0·36

R_F values are for edge of fluorescent flare.

At the end of four months extracts of tissue from a sample of the animals on each of the diets were subjected to chromatography. No obvious differences could be detected between any of the patterns obtained from the animals reared in the laboratory (Table 3), which were identical with those obtained from *T. pisana* of different ages, and from different geographical localities.

DISCUSSION

The method of paper chromatography applied to fresh tissue extracts developed by Buzzati-Traverso & Rechnitzer (1953) and the modification of it described here are both rapid and easy to carry out. The evidence so far available suggests that the patterns of such chromatograms are highly characteristic for a single species, and are uninfluenced by wide variations in environmental conditions or by age. The common European snail *T. pisana* is now widely dispersed along a narrow coastal belt in Western Australia over a distance of 1000 miles and is exposed to considerable differences in climate, soil and vegetation. Specimens taken from localities 300 miles apart showed no significant differences in the chromatographic pattern obtained from extracts of the foot musculature. Further, attempts to modify the pattern by rearing young snails on three different diets for four months in the laboratory gave rise to no significant differences in the patterns obtained. On the other hand, each of the seven separate species of land snail studied gave distinctively different patterns. On the basis of these preliminary observations, therefore, it seems clear that paper chromatography of simple tissue extracts offers a tool which is likely to be of considerable value to the taxonomist. With the further refinement of this kind of study and the identification or quantitative measurement of some of the components responsible for the patterns it is possible that degrees of relationship between closely related species may be assessed. If this is realized a new approach to the problems of phylogeny will be opened up which may be used in conjunction with the ecological, serological and biometrical methods of analysis now employed in evolutionary studies.

SUMMARY

1. Paper chromatography has been applied to fresh tissue extracts of the foot muscle of seven species of land snails. The characteristic ultraviolet fluorescence and absorption patterns obtained, using butanol:acetic acid:water as the solvent, are described. Each species gives a pattern which can be distinguished clearly from the others.

2. The effect on the patterns of variations in age for *T. pisana* and *H. aspersa* and of geographical locality and of three different diets under laboratory conditions for *T. pisana* has been studied. No significant differences were found in the characteristic pattern under any of the conditions studied.

3. The potential value of chromatography of fresh tissue extracts for systematic and phylogenetic investigations is discussed.

We are indebted to the University of Western Australia research grants committee for financial aid.

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The Effect of Ketone Bodies on the Carbohydrate Metabolism of Rat Diaphragm

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High blood levels of ketone bodies have been associated with 'toxicity' in diabetic acidosis and other acidotic states. This toxicity has never been adequately explained, particularly in chemical terms, and there have been only few experimental investigations of the problem. Nath & Brahamachari (1944) and Nath & Chakrabarti (1950) injected small doses of acetoacetate and β-hydroxybutyrate into rabbits over a prolonged period. A depletion of stored glycogen was found in the injected animals as compared with controls. Tidwell & Axelrod (1948) administered small quantities of acetoacetate to rats for a shorter time and observed no effect.

In a preliminary note (Chari & Wertheimer, 1953), we reported the effects of ketone bodies on carbohydrate metabolism, in particular at concentrations comparable with those found in diabetic acidosis. This condition was obtained in the *in vivo* experiments by a single large injection of one of the ketone bodies. For the *in vitro* experiments, an equivalent amount of the respective ketone body was added to the incubation medium.

The purpose of the work recorded here is to determine whether ketone bodies are merely end-products of abnormal metabolism or metabolites which during their accumulation influence normal metabolism (Parnes & Wertheimer, 1950).

EXPERIMENTAL

Animals

Albino male rats of an inbred strain, weighing 80–120 g. when 3–3·5 months old, were used. Food was withheld for 20 hr. before the experiment. The rats were anaesthetized with pentobarbitone and bled.

In vitro *experiments*

Incubation of the diaphragm was by the method described by Gemmill (1940). The medium consisted of 1 ml. phosphate-buffered saline containing 0·74% NaCl, 0·015% Na_2HPO_4 and 0·003% NaH_2PO_4, pH 7·4 (Tuerkischer & Wertheimer, 1948). The diaphragms and medium were put into 10 ml. Erlenmeyer flasks and gassed for 30 sec. with oxygen. The flasks were then placed in a water bath at 37° and shaken at the rate of 120 excursions/min. for approximately 2·5 hr.

Substrates. All substrates were used as sodium salts. Sodium acetoacetate was prepared according to Ljunggren (1924), and calculated as acetoacetic acid. Sodium β-hydroxybutyrate was a product of British Drug Houses Ltd. The concentrations of the substrates, given in the text as % (w/v), were equivalent to about 0·008 M.

Measurement of glycogen synthesis. The measurements were carried out according to Tuerkischer & Wertheimer (1948).

Measurement of glycogenolysis. These experiments were performed in the phosphate-buffered solution mentioned above, without adding glucose. When serum was used as the medium, it was freed of glucose by incubation with yeast for 10 min. at 37°.

Measurement of glucose utilization. The method described by Krahl & Cori (1947) and Perlmutter & Greep (1948) was used. Values of glycogen synthesis, glycogenolysis and glucose utilization are all expressed in mg. glucose/100 mg. wet tissue.

Measurement of fatty acid oxidation. Serum was chosen as a medium for the experiments on fatty acid oxidation, following the method of Wertheimer & Bentor (1952). Glucose was removed by fermentation with yeast and the ketone bodies were added. Serum without ketone bodies was employed as control. The diaphragm was incubated in this serum for 2·5 hr. at 37°. Fatty acid oxidation was expressed as mg. fatty acid/100 mg. wet tissue.

Respiration measurements. Determination of oxygen uptake was carried out by Warburg's direct method (Dixon, 1951), in phosphate-saline medium. Measurements were made over periods varying from 2 to 3 hr., and the average value for 1 hr. was calculated.

Measurement of glycogen synthesis in liver slices. Liver slices, prepared with a tissue slicer (Stadie & Riggs, 1944), were incubated for 3 hr. in 1 ml. of the medium described by Buchanan, Hastings & Nesbett (1949) or normal rat serum. The concentration of glucose was 0·5% and of acetoacetate, 0·1% or 0·5%. The flasks were gassed with 95% O_2+5% CO_2 and placed in a water bath at 37°.

In vivo *experiments*

In order to raise the level of ketone bodies *in vivo* several methods were employed:

(*a*) Ketone bodies in saline were administered intraperitoneally to rats fasted for 20 hr. The dose was 100 mg./100 g. body wt., since lesser quantities were without obvious effect. Control animals received saline and acetate.

(*b*) Diabetes was induced by intravenous injection of 5·5 mg. alloxan/100 g. body wt. Animals were used in various stages of diabetes ranging from severe coma to latent diabetes. They were fasted for 20 hr. before use.

(*c*) Rats were fasted for 1–7 days. The rats fasted for 7 days lost about 36% of their weight and were in poor condition.

(*d*) Rats were fed for 6–12 days on the following diet: 62% fat (margarine), 21% casein, 10% carbohydrate (starch), 4% salt mixture and 3% dried yeast. After the rats had been fasted for 20 hr. the experiments were carried out in the usual way.

Chemical determinations

Glycogen in the diaphragm was estimated by the method of Good, Kramer & Somogyi (1933); glucose, according to Somogyi (1937); total fatty acids, according to Stern & Shapiro (1953); ketone bodies, by the method of Engfeld, described by Rappaport (1949). The later values were given in mg. acetone/100 ml.

RESULTS

Effect of ketone bodies on the glycogen content of rat diaphragm with and without glucose

Addition of 0·1% acetoacetate to phosphate-buffered medium containing 0·1% glucose was accompanied by a decrease in the extent of glycogen synthesis. Addition of 0·05% acetone or 0·1% β-hydroxybutyrate had no effect on glycogen synthesis, while 0·25% β-hydroxybutyrate was stimulatory.

In the absence of glucose 0·1% acetoacetate had no effect on the rate of glycogenolysis. Under similar conditions, 0·1% β-hydroxybutyrate or 0·05% acetone markedly inhibited the rate of glycogenolysis (Fig. 1).

A similar effect was observed with liver slices. When incubated in normal rat serum or in the medium of Buchanan *et al.* (1949) containing 0·5% glucose, 0·1% acetoacetate did not significantly affect glycogen synthesis (0·199 ± 0·014 with acetoacetate, 7 expts.; 0·213 ± 0·013 without acetoacetate, 10 expts.). Addition of 0·5% acetoacetate, however, significantly decreased glycogen synthesis (0·173 ± 0·009, 8 expts.) in the medium of Buchanan *et al.* (1949).

This decrease of glycogen synthesis in the presence of acetoacetate will henceforth be called the 'acetoacetate effect'.

Effect of varying glucose and acetoacetate concentrations on glycogen synthesis by rat diaphragm

The 'acetoacetate effect' was found to be dependent on the relative concentrations of glucose and acetoacetate in the medium. The effect was diminished by (*a*) increasing the concentration of glucose relative to that of acetoacetate, or (*b*) decreasing the concentration of acetoacetate relative to that of glucose (Table 1).

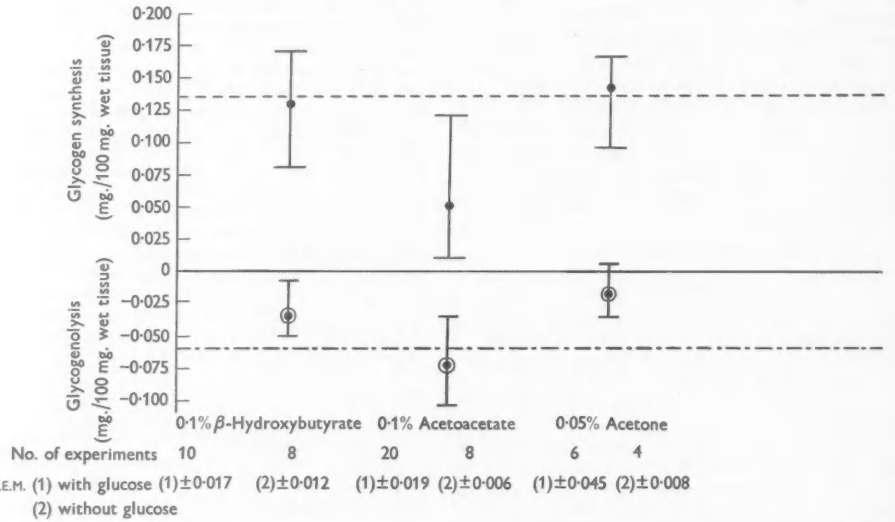

Fig. 1. Effect of ketone bodies on the glycogen content of rat diaphragm in presence and absence of glucose. – – –, glycogen content in the presence of 0·1% glucose but no other substrate; – · – ·, glycogen content without addition of any substrate. ϕ, Glycogen content when one substrate—but not glucose—is added. ϕ, Glycogen content when a substrate and glucose are added.

Table 1. *Effect of varying glucose and acetoacetate concentrations on glycogen synthesis of rat diaphragm*

The diaphragms were incubated at 37° for 2·5 hr. Glycogen synthesis is expressed as mg. glucose/100 mg. wet tissue ± standard error of the mean.

Varying glucose concentration				Varying acetoacetate concentration			
No. of measurements	Glucose (g./100 ml.)	Aceto-acetate (g./100 ml.)	Glycogen synthesis	No. of measurements	Glucose (g./100 ml.)	Aceto-acetate (g./100 ml.)	Glycogen synthesis
7	0·05	0	0·098 ± 0·012	—	—	—	—
7	0·05	0·10	0·007 ± 0·008	—	—	—	—
12	0·10	0	0·136 ± 0·011	12	0·10	0	0·136 ± 0·011
12	0·10	0·10	0·042 ± 0·007	5	0·10	0·025	0·125 ± 0·038
7	0·20	0	0·184 ± 0·029	7	0·10	0·05	0·076 ± 0·010
12	0·20	0·10	0·175 ± 0·014	12	0·10	0·10	0·042 ± 0·007

Table 2. *Effect of ketone bodies on glucose utilization of rat diaphragm*

The diaphragms were incubated at 37° for 2·5 hr. Final concentrations: glucose, acetoacetate and β-hydroxybutyrate, 0·1 g./100 ml.; acetone, 0·05 g./100 ml.

No. of measurements	Medium	Glucose utilization (mg./100 mg. wet tissue)
30	Glucose	0·50 ± 0·003
11	Glucose + β-hydroxybutyrate	0·54 ± 0·009
6	Glucose + acetone	0·52 ± 0·009
21	Glucose + acetoacetate	0·57 ± 0·004

Table 3. *Effect of ketone bodies on the oxygen uptake of rat diaphragm*

The diaphragms were incubated in phosphate-saline containing either 0·1 % acetoacetate or 0·1 % β-hydroxybutyrate with or without the addition of 0·1 % glucose; control contained either 0·1 % glucose alone or nil.

No. of measurements	Medium	Q_{O_2} (μl./mg. dry wt./hr.)
3	Nil	−6·31 ± 0·23
9	Glucose	−6·12 ± 0·10
6	Acetoacetate	−7·33 ± 0·10
6	Acetoacetate + glucose	−7·84 ± 0·12
5	β-Hydroxybutyrate	−6·44 ± 0·60
5	β-Hydroxybutyrate + glucose	−7·00 ± 0·43

Table 4. *Effect of ketone bodies on the oxidation of fatty acids*

The diaphragms were incubated at 37° for 2·5 hr. in glucose-free normal rat serum. Final concentrations of acetoacetate, and β-hydroxybutyrate 0·1 %, acetone, 0·05 %.

No. of measurements	Substrate	Fatty acid oxidation (mg./100 mg. wet tissue)
8	Nil	0·73 ± 0·007
7	Acetoacetate	0·60 ± 0·009
7	β-Hydroxybutyrate	0·31 ± 0·006
4	Acetone	0·28 ± 0·027

Effect of ketone bodies on the glucose utilization and oxygen uptake of rat diaphragm

Addition of 0·1 % acetoacetate caused a slight but significant increase in glucose utilization; 0·1 % β-hydroxybutyrate or 0·05 % acetone showed no such effect (Table 2).

The oxygen uptake was greater in the presence of 0·1 % acetoacetate than in the presence of 0·1 % glucose, and was greater still in the presence of both acetoacetate and glucose. β-Hydroxybutyrate behaved similarly to acetoacetate, but the effect was not so marked (Table 3).

Effect of ketone bodies on the oxidation of fatty acids by rat diaphragm

Oxidation of fatty acids was measured in normal rat serum after removal of glucose. Compared with the control value, acetoacetate caused only a slight decrease in the rate of oxidation, while β-hydroxybutyrate and acetone caused about 50 % inhibition (Table 4).

Influence of ketone bodies on glycogen synthesis and glucose utilization of rat diaphragm in the presence of insulin and adrenalin

Insulin (0·1 unit) increased glycogen synthesis by the rat diaphragm in the presence of glucose, as first reported by Gemmill (1940). Acetoacetate (0·1 %) diminished this stimulation, but was without effect on glucose utilization (Fig. 2). β-Hydroxybutyrate and acetone did not affect the stimulation of glycogen synthesis by insulin.

This effect of adrenalin on glycogen synthesis by rat diaphragm was not influenced by the addition of acetoacetate. When 0·1 mg./100 ml. adrenalin and 0·1 % glucose were present, a value of −0·003 ± 0·033 was obtained in six expts.; addition of 0·1 % acetoacetate gave a value of 0·015 ± 0·025 in 8 expts.

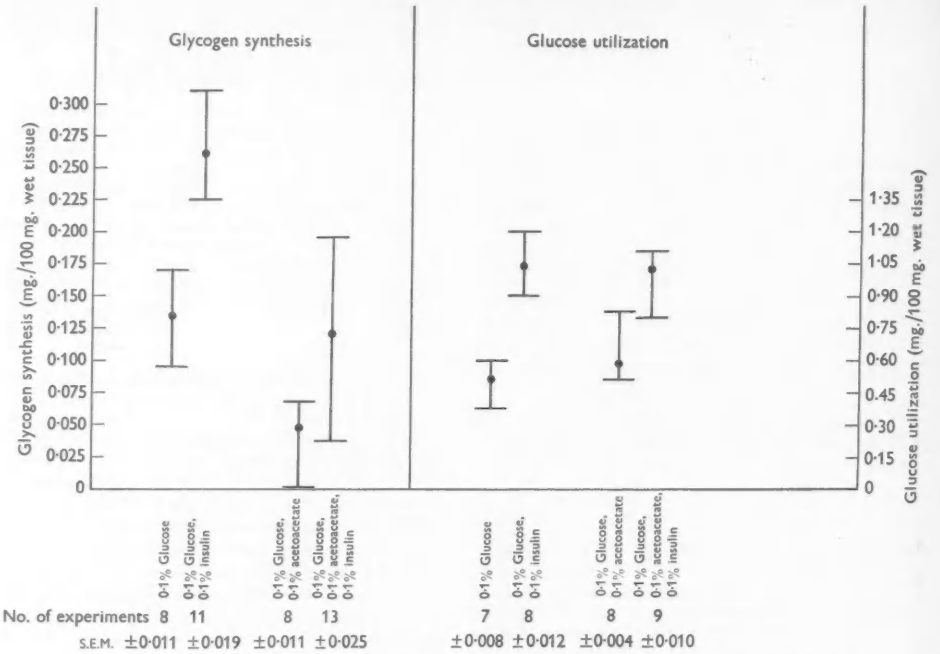

Fig. 2. Effect of acetoacetate on glycogen synthesis and glucose utilization in the presence of insulin.

Table 5. *Effect of β-hydroxybutyrate on the 'acetoacetate effect'*

Diaphragms were incubated at 37° for 2·5 hr. Glycogen synthesis is expressed as mg. glucose/100 mg. wet tissue. Glucose, acetoacetate and β-hydroxybutyrate all used at a final concn. of 0·1%. Glucose present in all flasks.

No. of measurements	Additions	Glycogen synthesis
6	Acetoacetate	0·053±0·013
10	β-Hydroxybutyrate	0·130±0·016
8	Acetoacetate + β-hydroxybutyrate	0·144±0·018

Effect of β-hydroxybutyrate on the 'acetoacetate effect' in diaphragms of normal and diabetic rats

β-Hydroxybutyrate (0·1%) in a medium containing 0·1% acetoacetate and 0·1% glucose diminished the 'acetoacetate effect' (Table 5).

β-Hydroxybutyrate (0·1%) in media containing diaphragms of diabetic rats caused an increase of glycogen synthesis in the presence of 0·1% glucose (0·149 ± 0·019 in 7 expts. with β-hydroxybutyrate; 0·104 ± 0·015 in 7 expts. without β-hydroxybutyrate). The acetone concentration in the blood was 19 ± 3 mg./100 ml.

A similar effect of β-hydroxybutyrate had already been observed after 0·2% was added to normal diaphragms.

Effect of methylene blue on glycogen synthesis and glucose utilization in presence and absence of acetoacetate

Methylene blue (0·0001 M) decreased glycogen synthesis and increased glucose utilization as acetoacetate had done. The effects of acetoacetate and methylene blue were not additive (Table 6).

'Acetoacetate effect' in rats in different stages of diabetes

The animals were divided into two groups: (1) blood-sugar levels of 200–500 mg./100 ml., (2) blood-sugar levels of 500–1000 mg./100 ml.

The 'acetoacetate effect' was clearly observed in the diaphragms of group (1) (0·065 ± 0·018 in the presence of acetoacetate, 8 expts., compared with 0·114 ± 0·026 in the absence of acetoacetate, 8 expts.). The acetone concentration in the blood was 13 ± 2 mg./100 ml. The 'acetoacetate effect' was absent from the diaphragms of group (2) (0·099 ± 0·028 with acetoacetate, 6 expts., compared with

Table 6. *Effect of methylene blue on glycogen synthesis and glucose utilization in presence and absence of acetoacetate*

The diaphragms were incubated at 37° for 2·5 hr. Glycogen synthesis and glucose utilization expressed as mg. glucose/100 mg. wet tissue. Glucose (0·1 %) present in all flasks. Additions: acetoacetate (0·1 %) or methylene blue (0·0001 M).

No. of measurements	Additions	Glycogen synthesis	Glucose utilization
10	Methylene blue	−0·074±0·013	0·56±0·005
6	Methylene blue + acetoacetate	−0·046±0·010	0·55±0·006
6	Nil	0·140±0·010	0·48±0·004
8	Acetoacetate	0·055±0·024	0·55±0·005

Table 7. *Effects of injected acetoacetate, β-hydroxybutyrate, acetate and saline of rat diaphragm on carbohydrate metabolism*

Diaphragms were incubated at 37° for 2·5 hr. Glycogen synthesis and glucose utilization are expressed as mg. glucose/100 mg. wet tissue. Acetone bodies are expressed as mg. acetone/100 ml. Glucose level is given as mg./100 ml.

No. of measurements	Substance injected	Time after injection (hr.)	Blood acetone concn.	Blood glucose concn.	Glucose utilization	Glycogen synthesis
14	Saline	0–2	7·4±1	82±7	0·47±0·003	0·159±0·014
13	Acetoacetate	0–2	27·1±1	62±5	0·43±0·007	0·105±0·015
5	β-Hydroxybutyrate	0–2	18·0±2	76±5	0·45±0·003	0·120±0·013
3	Acetate	0–2	8·0±2	89±5	0·44±0·004	0·155±0·015
5	Saline	6	6·3±1	84±7	0·45±0·002	0·155±0·028
5	Acetoacetate	6	11·0±2	80±6	0·53±0·007	0·120±0·023
8	Saline*	18–20	12·1±1	70±6	0·40±0·005	0·070±0·019
9	Acetoacetate*	18–20	13·7±1	76±5	0·49±0·004	0·084±0·012

* These experiments were carried out after 36 hr. fasting.

0·065±0·012 without acetoacetate, 6 expts.). The acetone concentration in the blood was 23±3 mg./100 ml.

'Acetoacetate effect' in starved rats

Rats starved 5–7 days lost about 32–38 % of their initial weight. The 'acetoacetate effect' was observed in rats fasted for 1–3 days but was not seen in those starved for 5–7 days (0·085±0·021 with acetoacetate, 6 expts., compared with 0·097±0·017 without acetoacetate, 6 expts.). The acetone concentration in the blood was 11±3 mg./100 ml.

'Acetoacetate effect' in rats fed a high-fat diet

After 12 days on the high-fat diet, the animals were killed and their diaphragms found to exhibit a normal 'acetoacetate effect' (0·060±0·017 with acetoacetate in 5 expts. compared with 0·139±0·023 without acetoacetate in 5 expts.). The acetone concentration in the blood was 9·3 mg./100 ml. as compared with 5·8 mg./100 ml. after 1 days fasting.

Influence of injected acetoacetate on glycogen synthesis and glucose utilization by rat diaphragm in vitro*; comparison with other injected substances*

A single injection of acetoacetate (100 mg./100 g. body weight) was followed by a rise in blood acetone bodies. *In vitro* the diaphragms of these rats showed a decreased glycogen synthesis compared with those which had been injected with equivalent amounts of acetate or with saline. In general, the higher the level of blood acetone *in vivo*, the greater the depression of glycogen synthesis *in vitro* (Table 7). This effect was still noticeable up to 6 hr. after injection, at which time the concentration of acetone bodies was approaching normal levels. The effect after 6 hr. was not significant.

The injection of equivalent doses of β-hydroxybutyrate also caused a decrease in glycogen synthesis by the rat diaphragm, but the effect was of doubtful significance ($P = 0{\cdot}05$).

In Table 7 it is seen that a single injection of acetoacetate of 100 mg./100 g. body weight, caused a decrease in the blood-glucose level, thus confirming the observation of Tidwell & Axelrod (1948). On the other hand, insignificant effects were produced by the other substrates administered. These injections were followed by an alkalosis which disappeared after 3 hr.

In another series of experiments an injection of acetoacetate was followed by one of β-hydroxybutyrate. The latter cancelled the 'acetoacetate effect' (0·015±0·015, 13 expts., when acetoacetate alone was administered; 0·120±0·013, 5 expts., when β-hydroxybutyrate was administered; and 0·133±0·028, 4 expts., when acetoacetate was followed by one of β-hydroxybutyrate).

It should be noted that a single injection of 100 mg. acetoacetate frequently caused convulsions and restlessness, as reported by Nath & Sahu (1952). These effects were seldom observed after an injection of β-hydroxybutyrate.

DISCUSSION

The present investigation shows that acetoacetate is the only ketone body which has a significant effect on glycogen synthesis in the diaphragm and liver slices of the rat, at concentrations comparable with those found in diabetic coma. This effect is noticed only within a small range of glucose concentrations relative to the concentration of acetoacetate. Similar interdependence has been found for the action of acetate on glycogen synthesis by rat diaphragm (Parnes & Wertheimer, 1950). The absence of an 'acetoacetate effect' at other relative concentrations of glucose is in agreement with the experiments of Nagler (1952) and of Tidwell, Nagler & Dunkelberg (1953).

Acetoacetate, but not β-hydroxybutyrate or acetone, causes in the rat diaphragm a slight increase of glucose utilization and oxygen consumption.

Inhibition of insulin action has been reported by Guest, Mackler & Knowles (1952) in acidotic dogs and by Duncan (1952) in acidotic diabetic patients. In our *in vitro* experiments, carried out in the presence of acetoacetate, an inhibition of insulin action has also been found. The absolute increment of glycogen synthesis due to insulin is decreased by acetoacetate (Fig. 2). However, when taking into account the initial lower glycogen synthesis, caused by acetoacetate in the absence of insulin, the relative augmentative action of insulin is not affected by acetoacetate.

In vivo experiments confirm the *in vitro* results. A single injection of acetoacetate (100 mg./100 g. body weight) causes an increased concentration of ketone bodies in the blood and a depression of glycogen synthesis by the rat diaphragm *in vitro*. This effect has been present up to 6 hr. after acetoacetate administration, at which time the concentration of blood ketone bodies has been approaching normal levels.

It is possible that acetoacetate plays the role of a hydrogen acceptor and increases glucose catabolism, concomitantly depressing glycogen synthesis. In agreement with this suggestion, β-hydroxybutyrate injected after acetoacetate or added *in vitro*, inhibits the 'acetoacetate effect' on glycogen synthesis.

In conclusion, it may be stated that acetoacetate is the only toxic ketone body. Under normal, non-pathological conditions the appearance of β-hydroxybutyrate and acetone as products of acetoacetate metabolism neutralizes the toxic 'acetoacetate effect'. In cases of diabetic coma or prolonged starvation, acetoacetate is produced in larger quantities than normal. Since diabetic animals utilize ketone bodies as rapidly as normal animals (Barnes, Drury, Greeley & Wick, 1940), a surplus of ketone bodies accumulates, acetoacetate being the first to appear. This surplus of acetoacetate decreases glycogen synthesis to a certain extent. Further addition of acetoacetate *in vitro* to the diaphragm of an animal suffering from diabetic coma or prolonged starvation will not increase the effect.

On the basis of our experiments it is probable that acetoacetate at high concentrations is one of the factors causing derangement of carbohydrate metabolism.

SUMMARY

1. The effect of ketone bodies on the *in vitro* carbohydrate metabolism of rat diaphragm has been investigated.

2. Acetoacetate depressed glycogen synthesis in rat diaphragm and slightly increased glucose utilization. These effects were dependent on the relative concentrations of acetoacetate and glucose.

3. Acetoacetate depressed glycogen synthesis in rat-liver slices. As with the rat diaphragm the effect was dependent on the relative concentrations of acetoacetate and glucose.

4. β-Hydroxybutyrate at a concentration equivalent to that of acetoacetate was without effect on the glycogen synthesis and the glucose utilization of rat diaphragm. Higher concentrations resulted in an increased glycogen synthesis. Addition of β-hydroxybutyrate to a medium containing acetoacetate cancelled the 'acetoacetate effect'.

5. Methylene blue, like acetoacetate, caused a decrease in glycogen synthesis and an increase in glucose utilization.

6. No 'acetoacetate effect' was found with diaphragms of severely diabetic rats, or of rats fasted for 5–7 days. Addition of β-hydroxybutyrate caused an increase of glycogen synthesis by the diaphragms of diabetic rats.

7. The oxidation of serum fatty acids by rat diaphragm was markedly depressed by β-hydroxybutyrate and acetone but only slightly by acetoacetate.

8. The stimulation by insulin of glycogen synthesis from glucose by rat diaphragm was diminished by the addition of acetoacetate.

9. Administration of acetoacetate *in vivo* depressed glycogen synthesis by the isolated rat diaphragm. The influence of acetoacetate persisted up to 6 hr. after injection. Administration of β-hydroxybutyrate *in vivo* following acetoacetate cancelled the 'acetoacetate effect'.

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The Component Acids of the Fats of Some Indian Fresh-water Fishes

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The component acids of fats from different parts of bhakur (*Catla buchanani*) were investigated by Pathak & Agarwal (1952). The fats obtained from the body and viscera of two other species of Indian fresh-water fishes have now been studied.

EXPERIMENTAL

The fresh-water fishes used were rohu (*Labeo rohita*) and nain (*Cirrhina mrigala*) caught in the River Ganges near Banaras. The body and viscera (excluding the liver) were separately cut into small pieces and exhaustively extracted with hot acetone. The extracted material was taken up in light petroleum (b.p. 40–60°), the aqueous fraction was separated and the lipids were recovered and dried at 100° under reduced pressure. The viscera of these fish contained nearly 45% of fat. The fats were kept in 10 vol. of acetone at 0° for 4 weeks to separate the phosphatides, if present, from the glycerides.

The fats were hydrolysed, and the mixed fatty acids resolved into groups of simpler mixtures of acids by the Li salt–acetone method (Tsujimoto, 1920; Tsujimoto & Kimura, 1923), followed by further resolution of the insoluble fraction by the Pb salt–ethanol method as modified by Hilditch (1949). Each group of acids was separately converted into methyl esters, with the precautions recommended by Bjarnson & Meara (1944), and fractionated at approx. 0·1 mm. pressure through an electrically heated, packed column (Longenecker, 1937). The compositions of the ester fractions were calculated from the iodine values and the saponification equivalents by the method of Hilditch (1949). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturation (e.g. −2·0, mono-ethenoid), was determined as usual by interpolation or extrapolation from the respective ester fractions in each group. The compositions of the original fats were obtained from those of the separated groups of fatty acids.

RESULTS

The results of the preliminary fractionation of 180 g. mixed body fatty acids and 175 g. mixed visceral fatty acids of rohu are given in Table 1. This table also gives results for 162·9 g. mixed body fatty acids and 134·7 g. mixed visceral fatty acids of nain.

The component acids of each group and of the whole of the body and the visceral fats are recorded in Tables 2 and 3 for rohu and in Tables 4 and 5 for nain.

DISCUSSION

Rohu body and visceral fats

These fats show similarities to typical fats of fresh-water fishes in having unsaturated C_{18} acids as the predominant group, palmitic acid being the major saturated acid component. There are traces of unsaturated C_{24} acids (Table 2), and this has also been observed in the body fat of bhakur, another species of Indian fresh-water fish (Table 6).

Table 1. *Fractionation of the fats of rohu and nain fish*

Group	Rohu fats				Nain fats			
	Body		Viscera		Body		Viscera	
	Wt. (%)	Iodine value	Wt. (%)	Iodine value	Wt. (%)	Iodine value	Wt. (%)	Iodine value
A. Pb salt, ethanol-insoluble	28·3	9·9	43·3	3·6	25·9	6·6	27·3	6·3
B. Li salt, acetone-insoluble, but Pb salt, ethanol-soluble	52·2	96·3	33·5	165·2	57·2	117·1	59·1	109·7
C. Li salt, acetone-soluble	19·5	238·0	23·2	213·0	16·9	167·8	13·6	163·9

Table 2. *Component acids of rohu body fat*

Acid	Component acids in groups (as % of total)			Sum (%)	Total as % of whole fat, excluding unsaponifiable fraction	
	A	*B*	*C*		% (w/w)	% (moles)
Myristic	1·2	·2	—	3·4	3·4	4·0
Palmitic	19·0	2·1	—	21·1	21·2	22·7
Stearic	5·2	6·3	—	11·5	11·5	11·5
Arachidic	—	0·4	—	0·4	0·5	0·4
Unsaturated fractions						
C_{14}	Trace	3·6	—	3·6	3·7	4·5
C_{16}	1·2	5·8	1·2	8·2	8·1	8·5
C_{18}	1·7	24·3	6·2	32·2	32·2	31·5
C_{20}	—	7·4	5·0	12·4	12·4	11·1
C_{22}	—	—	6·7	6·7	6·7	5·5
C_{24}	—	—	0·3	0·3	0·3	0·3
Unsaponifiable fraction	Trace	0·1	0·1	0·2	—	—
Mean unsaturation of fractions						
C_{14}	−2·0	−2·0	—	−2·0	—	—
C_{16}	−2·0	−2·0	−3·3	−2·2	—	—
C_{18}	−2·0	−3·3	−4·6	−3·3	—	—
C_{20}	—	−4·7	−6·0	−5·2	—	—
C_{22}	—	—	−6·9	−6·9	—	—
C_{24}	—	—	−8·0	−8·0	—	—

Table 3. *Component acids of rohu visceral fat*

Acids	Component acids in groups (as % of total)			Component acids in whole fat	
	A	*B*	*C*	% (w/w)	% (moles)
Myristic	1·6	—	—	1·6	2·0
Palmitic	23·4	2·6	—	26·0	28·0
Stearic	13·3	1·3	—	14·6	14·1
Arachidic	3·1	Trace	—	3·1	2·7
Unsaturated fractions					
C_{14}	Trace	—	0·7	0·7	0·9
C_{16}	0·7	4·1	3·8	8·6	9·4
C_{18}	0·7	22·0	7·6	30·3	29·8
C_{20}	0·5	3·5	5·6	9·6	8·6
C_{22}	—	—	5·5	5·5	4·5
Unsaponifiable fraction	Trace	Trace	Trace	—	—
Mean unsaturation of fractions					
C_{14}	−2·0	−2·0	−2·0	−2·0	—
C_{16}	−2·0	−2·0	−3·6	−2·7	—
C_{18}	−2·0	−3·2	−4·9	−3·6	—
C_{20}	−2·0	4·0	−8·0	−6·3	—
C_{22}	—	—	−9·0	−9·0	—

Table 4. *Component acids of nain body fat*

Acids	Component acids of groups (%) A	B	C	Sum (%)	Total as % of whole fat excluding unsaponifiable fraction % (w/w)	% (moles)
Myristic	1·7	0·2	—	1·9	1·9	2·2
Palmitic	19·2	2·1	—	21·3	21·4	22·2
Stearic	3·1	—	—	3·1	3·1	2·9
Unsaturated fractions						
C_{14}	Trace	3·0	0·6	3·6	3·7	4·3
C_{16}	1·1	27·7	3·7	32·5	32·6	34·0
C_{18}	0·7	22·2	6·5	29·4	29·5	27·9
C_{20}	—	1·9	3·0	4·9	5·0	4·3
C_{22}	—	—	2·8	2·8	2·8	2·2
Unsaponifiable fraction	0·1	0·1	0·3	0·5	—	—
Mean unsaturation of fractions						
C_{14}	−2·0	−2·0	−2·0	−2·0	—	—
C_{16}	−2·0	−2·1	−3·4	−2·2	—	—
C_{18}	−2·0	−3·5	−4·3	−3·6	—	—
C_{20}	—	−4·5	−5·2	−4·9	—	—
C_{22}	—	—	−6·0	−6·0	—	—

Table 5. *Component acids of nain visceral fat*

Acids	Component acids of groups (%) A	B	C	Total as % of whole fat excluding unsaponifiable fraction % (w/w)	% (moles)
Myristic	1·9	3·9	—	5·7	6·6
Palmitic	17·6	2·4	—	20·1	20·7
Stearic	5·6	0·1	—	5·7	5·3
Arachidic	0·5	—	—	0·5	0·4
Unsaturated fractions					
C_{14}	Trace	2·7	1·5	4·2	4·9
C_{16}	0·4	21·8	4·2	26·5	27·5
C_{18}	1·0	26·0	5·3	32·3	30·3
C_{20}	0·2	2·1	2·6	5·0	4·3
Unsaponifiable fraction	0·1	0·1	Trace	—	—
Mean unsaturation of fractions					
C_{14}	−2·0	−2·0	−2·0	−2·0	—
C_{16}	−2·0	−2·0	−3·5	−2·2	—
C_{18}	−2·0	−2·6	−4·6	−2·9	—
C_{20}	−2·0	−3·1	−6·0	−4·6	—

The composition of the visceral fat is somewhat different from that of the body fat in both saturated and unsaturated fractions (Tables 2 and 3). Unsaturated C_{24} acids are absent. Similar small variations in the proportions of various component acids in fats from different tissues of the same fish have also been observed, e.g. in pike (Lovern, 1932*a*) sturgeon (Lovern, 1932*b*) and salmon (Lovern, 1934, 1936*a*, 1937).

The more remarkable feature of these two fats is, however, their high saturated acid contents (36·6 and 45·3 % respectively). The body and visceral fats of bhakur contain 38·4 and 37·4 % of saturated acids respectively (Pathak & Agarwal, 1952). Except for the doubtful case of the Chinese fan fish (Tsujimoto, 1916), the highest percentage of saturated acids recorded in other fish fats is about 28·0 % in groper liver (Shorland & Hilditch, 1939) and in the heart and spleen of tunny from the Atlantic Ocean (Lovern, 1936*b*). The proportion of stearic acid (11·5 and 14·6 % in body and visceral fats) is still more remarkable, as the highest percentage previously reported (excluding the Chinese fan fish) is about 8 % in tunny (Lovern, 1936*b*). The total C_{18} acid content in these fats is similar (43·7 and 44·9 % of the total), and is of the usual order for the fat of fresh-water fishes.

This suggests that an otherwise normal fish fat has undergone bio-hydrogenation with the appearance of larger amounts of stearic acid and a consequent increase in the total saturated acid.

Table 6. *Fatty acid compositions of fresh-water and marine fish fats from different parts of the same fish*

All values are as percentage of total. The figures in parentheses give the mean unsaturation of the component.

Acids	Herring body*	Herring viscera†	Bhakur body‡	Bhakur viscera‡	Bhakur liver‡	Nain body	Nain viscera	Rohu body	Rohu viscera
Myristic	8·3	5·8	2·9	2·8	0·6	1·9	5·7	3·4	1·6
Palmitic	12·1	15·7	29·0	25·3	19·6	21·4	20·1	21·2	26·0
Stearic	0·3	2·8	6·5	7·9	6·4	3·1	5·7	11·5	14·6
Arachidic	—	0·3	—	1·4	3·1	—	0·5	0·5	3·1
Unsaturated fractions									
C_{14}	0·5 (−2·0)	1·4 (−2·0)	0·2 (−2·0)	1·4 (−2·0)	—	3·7 (−2·0)	4·2 (−2·0)	3·7 (−2·0)	0·7 (−2·0)
C_{16}	6·4 (−3·4)	10·5 (−2·5)	25·3 (−2·7)	10·0 (−2·7)	7·0 (−2·0)	32·6 (−2·2)	26·5 (−2·2)	8·1 (−2·2)	8·6 (−2·7)
C_{18}	31·0 (−4·5)	31·8 (−2·6)	17·9 (−2·5)	37·2 (−2·5)	25·4 (−2·7)	29·5 (−3·6)	32·3 (−2·9)	32·2 (−3·3)	30·3 (−3·6)
C_{20}	28·3 (−5·5)	22·4 (−7·1)	7·5 (−5·7)	11·9 (−5·7)	26·5 (−4·8)	5·0 (−4·9)	5·0 (−4·0)	12·4 (−5·2)	9·6 (−6·3)
C_{22}	23·1 (−4·6)	9·3 (−10·5)	10·1 (−9·0)	2·1 (−9·0)	9·4 (−6·5)	2·8 (−6·0)	—	6·7 (−6·9)	5·5 (−9·0)
C_{24}	—	—	0·6 (−11·0)	—	2·6 (−8·0)	—	—	0·3 (−8·0)	—

* Lovern (1938*a*). † Hilditch & Pathak (1948). ‡ Pathak & Agarwal (1952).

Nain body and visceral fats

Nain belongs to a different genus from rohu, and this is clearly reflected in the nature of its body and visceral fats. Differences are apparent in the lower stearic acid content, in the lower proportions of saturated acids, and more especially in the unsaturated C_{16} acids (Tables 2–5). Unsaturated C_{16} acids comprise only 8·1 and 8·6 % of the rohu body and visceral fats but form major components in the fats of nain, being 32·6 and 26·5 % respectively. Palmitic acid, which constitutes 21·4 and 20·1 % respectively of the body and visceral fats of nain, together with unsaturated C_{16} (32·6 and 26·5 %) and C_{18} (29·5 and 32·3 %) acids, account for nearly 80–85 % of the total component acids of these nain fats. The proportions of C_{20-22} acids (7·8 and 5·0 %) in these fats are much smaller than those in rohu fats.

The percentages of the various component acids are different in the body and in the visceral fat; the chief component acid in the fat of nain body is C_{16} while in that of viscera it is C_{18}. Thus these fats are similar in this respect to the body and visceral fats of bhakur (Table 6). There are variations in the composition of fats from different parts of nain, but these are not so outstanding as in the case of fats from different tissues of bhakur and herring (Table 6). Fats of the latter fish are typically marine in nature and are included in the table as they show clearly the differences between fats from marine and fresh-water sources. There are much increased proportions of the C_{20} and C_{22} groups of unsaturated acids with corresponding reductions in the amount of unsaturated C_{16} and C_{18} acids. The high percentage of stearic acid and total content of saturated acid are not so marked in the case of nain fats as in rohu fats, and bio-hydrogenation has evidently not proceeded to a great extent in this case.

The comparatively saturated nature of these fats may be related to the fact that the fishes belong to warm tropical waters, since Lovern has observed that in the cases of eels (Lovern, 1938*b*) and tunny (Lovern, 1936*b*) there is a definite tendency towards the deposition of a less unsaturated fat at a higher temperature.

Rapson, Schwartz & Van Rensburg (1945) noted that head and body tissues of groper contained about 4 times as much fat as the mesentrium, and Pathak & Agarwal (1952) found that in bhakur the main fat depot was in the viscera, which contained about 45 % fat. Our results show that the viscera of rohu and nain also serve as the main fat depot, and contain on the average 45 % of fat.

SUMMARY

1. The composition of body and visceral fats from rohu (*Labeo rohita*) and nain (*Cirrhina mrigala*) have been studied. Preliminary separations of the fatty acids into groups differing in unsaturation

were carried out using the lithium and lead salts in acetone and ethanol respectively. Their compositions were studied by the ester-fractionation procedure.

2. The fishes rohu and nain belong to different genera, and significant differences have been found in the proportions and degree of unsaturation of their C_{16}, C_{18}, C_{20} and C_{22} groups of unsaturated acids, and also in the amounts of total saturated acids and of stearic acid.

3. The high saturated acid content of the fats is remarkable, especially in fats from rohu. The increase in the percentage of stearic acid seems to have been at the cost of unsaturated C_{18} acids, and thus may indicate bio-hydrogenation. Small differences in the composition of the fats from body and viscera of the same fish have been observed in both species.

4. The fat content of the viscera of these fish is about 45%.

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The Organic Constituents of Mammalian Compact Bone

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The inorganic matter which characterizes bone as a tissue has been studied by a large number of workers (Neuman & Neuman, 1953). On the other hand, the material composing the so-called 'organic matrix' has received very little attention.

Collagen, the principal organic constituent of bone, has for a long time been assumed to be similar to the collagen of the softer connective tissues (Huggins, 1937), although hardly anything was known about it, except that under mild hydrolytic conditions, it gave rise to gelatin. Quite recently Neuman (1949), using microbiological methods of analysis, has shown that the amino acid composition of collagen from bone does not differ significantly from that of collagen in other tissues. The same conclusion has been reached by Rogers, Weidmann & Parkinson (1952). Randall, Fraser, Jackson, Martin & North (1952) have examined collagens from a number of tissues, including bone, with the electron microscope, and have found typical non-branching striated fibrils in all specimens.

Hawk & Gies (1901) isolated a mucopolysaccharide complex which they termed 'osseomucoid' from ox bone, and Seifert & Gies (1904) showed that it was invariably present, as a minor constituent, in the bones of a number of mammalian species. Hisamura (1938) separated osseomucoid into two components, one of which contained a hexosamine and a hexuronic acid and the other a hexosamine and galactose. Two hexosamines, glucosamine and galactosamine, were found in osseomucoid by Masamune, Yosizawa & Maki (1951), who separated them by paper chromatography. Rogers (1949) reported the presence of 0·1–0·25% of hexosamines and 2–4% of reducing substances in human bone and later (Rogers, 1951) isolated a substance containing equimolar weights of a hexosamine, a hexuronic acid and the hydrogen sulphate group.

The presence in bone of two proteins, one soluble in cold water, and similar in composition to collagen, and a second which resists solution in hot water, has been shown by Rogers *et al.* (1952).

The citrate content of bone has been extensively examined by Dickens (1941). This ion is generally considered to be intimately associated with the inorganic crystal lattice (Neuman & Neuman, 1953).

The object of the present study was to obtain more information about the composition of compact bone tissue. Ox-femur bone, which was readily freed from extraneous tissues, was powdered by a method involving negligible chemical degradation. It was then separated into its known constituents by a variety of methods. Prolonged extraction with water at 100° dissolved the principal organic constituent, collagen, by converting it into gelatin. The residue from this operation consisted of inorganic matter with a small amount of a protein which resisted solution in boiling water. A little of the inorganic matter dissolved during the hot-water extraction. Treatment of bone powder with dilute hydrochloric acid dissolved the inorganic matter leaving the organic matrix. A mucopolysaccharide-protein complex was separated from this organic portion by extraction with lime water. The residue, which consisted mainly of protein, was extracted with hot water to dissolve the collagen and leave the water-resistant protein. By carrying out these processes quantitatively a chemical 'balance sheet' was drawn up to show how far the entire composition of bone was accounted for in terms of its known constituents.

In the second part of this investigation the detailed composition of the organic constituents of bone was studied using analytical techniques. In particular, the amino acid composition of the protein involved in the mucopolysaccharide complex was determined quantitatively, and the identity of the components of the polysaccharide portion tentatively established.

EXPERIMENTAL

Materials

Compact bone tissue. The epiphyses of ox femora were sawn off leaving the straight centre portions of the diaphyses about 10 cm. in length. The fatty marrow was completely scooped out and small areas of spongy bone, inside the tube of compact bone, were scraped away with a scalpel. The periosteum was similarly scraped off while wet and any areas of discoloured bone, where ligaments had been attached, were removed. The remaining tissue consisted entirely of compact bone which was completely white, dense and hard.

Bone powder. The tubes of compact bone were turned in a lathe using a light cut. The light, friable turnings were collected on a clean cloth and reduced to a powder in a high speed disintegrator. The powder, which passed through a 100-mesh test sieve, was spread out in a thin layer and exposed to the air for 48 hr. to allow the moisture content to reach an equilibrium value.

Bone collagen (containing water-resistant protein). Sulphur dioxide was passed into a mechanically stirred suspension of 50 g. of bone powder in 1 l. of water until pH 2·5 was reached. The suspension was stirred for 30 min., allowed to settle and the supernatant liquid removed by suction. More water was added and the treatment with sulphur dioxide repeated. The residue was washed twice with water and treated with 10% NaCl solution at pH 7, overnight, to remove albumins and globulins. After further washing, the residue was treated overnight with 10% $CaCl_2$ at pH 9 to remove the mucopolysaccharide fraction (Partridge, 1948*a*). The residue from this operation was washed successively with water and acetone and dried in a vacuum desiccator. The protein, which was a finely divided white powder, was exposed to the air in a thin layer to allow the moisture content to reach equilibrium. (Moisture content, 15·25; ash, 0·08; total N, 18·26% on dry, ash-free basis.)

Mucopolysaccharide-protein complex. 'Osseomucoid' was prepared from ox femora using the lime water extraction method of Hawk & Gies (1901). The preliminary demineralization of the bone was carried out in a refrigerator at 2° to minimize possible degradation. Excess of $Ca(OH)_2$, above the amount required to prepare half-saturated lime water was added to allow for the buffering capacity of the protein. It was found necessary to add a slight excess of acid to the filtered lime-water extract to obtain the mucopolysaccharide-protein complex in a form which could be readily filtered. Filtration was carried out rapidly, the precipitate being washed successively with acetone and ether, since exposure of the white powder to the air, while moist, resulted in its conversion into a brown gum.

Osseomucoid was also prepared from commercially demineralized bone (ossein).

Water-resistant protein. Shavings of demineralized bone were autoclaved with a tenfold excess of water for 3 hr. at 15 lb./in.^{2} pressure. The residue was filtered from the brown supernatant liquid and again autoclaved for 2 hr., after more water had been added. The residue was filtered off, washed successively with hot water, acetone and ether and dried in a vacuum desiccator.

Analytical methods

Moisture. The loss of weight on drying a thinly spread layer of the powdered material at 105° for 24 hr. was used to calculate the moisture content.

Ash. The material was gently ignited in a platinum dish, then heated for 2 hr. at 550° and the residue weighed.

Total nitrogen. This was determined by the micro-Kjeldahl method, using a modification of the procedure of Chibnall, Rees & Williams (1943). The $CuSO_4$ in the catalyst mixture was replaced by an equal weight of $HgSO_4$, which reduced the digestion time necessary for maximum recovery of nitrogen, from 8 to 2 hr. A portion of the diluted solution was made alkaline with 30% NaOH, 5% $Na_2S_2O_3$ solution and the ammonia was steam distilled into 1% boric acid solution (Yuen & Pollard, 1953). This solution was titrated with 0·01 N-HCl using an ethanolic solution of 0·025% methyl red and 0·016% methylene blue as indicator.

Analysis of whole bone tissue. The oven-dry bone was extracted with ether and then with a large excess of water at 100° for 24 hr. The residue, consisting of inorganic matter, and water-resistant protein, was filtered on a sintered glass crucible, washed, dried at 105° and weighed. The total N content of the dry residue was determined and used to calculate the weight of resistant protein present, assuming that it contained 16·0% N. The water extract was evaporated to dryness and the residue, which consisted mainly of degraded gelatin, was dried at 105°, weighed and finally ashed to obtain the weight of soluble inorganic matter.

Sulphate sulphur. This was determined by the method of Bowes & Cave (personal communication) in HCl hydrolysates of the mucopolysaccharides.

Hexosamines. The combined glucosamine and galactosamine content was determined by the method of Elson & Morgan (1933) as modified by Bowes & Cave (personal communication), except that the heating with acetylacetone was carried out in stoppered flasks for 50 min. instead of in open test tubes for 20 min. to improve the colour yield.

Later, glucosamine and galactosamine were independently determined after chromatographic separation, by the ninhydrin method of Moore & Stein (1948).

Uronic acid. The modification of Tollen's test, due to Maughan, Evelyn & Browne (1938) was used.

Total reducing sugars. These were determined by the modification, worked out by Heidt, Southam, Benedict & Smith (1948) of the micro-volumetric method of Somogyi (1937).

Hydrolysis. Materials to be examined for amino acid composition were heated for 24 hr. with a 100-fold quantity of 20% (w/w) HCl in sealed glass ampoules (Macpherson, 1946). The HCl was subsequently removed by evaporation in a vacuum desiccator containing NaOH and P_2O_5.

Where hexosamines were determined by the method of Elson & Morgan (1933), the time of heating was reduced to 8 hr. and the acid concentration to 4 N.

The hydrolysates for examination of the sugar residues by paper chromatography were prepared by heating with N-H_2SO_4 at 100° for 5 hr. (Yosizawa, 1950).

Paper chromatography. The separation of both amino acids and sugars was carried out on Whatman no. 1 filter paper (Brimley & Barrett, 1953).

The solvents used for separation of amino acids were phenol:water (ammonia) and *n*-butanol:acetic acid:water (4:1:5, v/v) (Partridge, 1948*b*). Since the R_F values of the amino acids are low in the latter solvent, increased resolution of the slower-moving ones was obtained by returning the paper to the tank for further development, after drying the paper in the usual way at the end of the first development. Up to four successive developments have been given by this means, at the end of which the spots remained sharp. Ethanolic ninhydrin (0·1%) was used as spray reagent.

For the separation of sugars and substituted sugars the miscible solvent mixtures *n*-butanol:pyridine:water (5:3:2, v/v) of Chargaff, Levine & Green (1948) and *n*-butyl acetate:acetic acid:water (3:2:1, v/v) of Yosizawa (1950) were employed. The latter solvent was developed by Yosizawa for the separation of glucosamine and galactosamine, but is found not to be entirely satisfactory since the separation of these spots is small even when the solvent front is allowed to move much further than the distance specified. Since the hexosamine spots frequently tended to tail, an elongated, diffuse spot resulted instead of two distinct spots, when both amino sugars were present. Aniline hydrogen phthalate (Partridge, 1949) was found to be the most useful of a number of spray reagents examined. Hexosamines gave no colour after 5 min. heating and a chocolate brown after 30 min. heating. Separate spraying with alkaline acetylacetone solution and *p*-dimethylaminobenzaldehyde in HCl:ethanol (Partridge, 1948*b*) was used to confirm the position of the hexosamine spots.

Quantitative amino acid analysis. This was carried out as by Moore & Stein (1951), the amino acids being separated on columns of Dowex 50 resin, 200 to 400-mesh, 12% cross-linking. A specially constructed drop-counting fraction collector was used for collecting 1 ml. (20 drop) fractions.

Difficulty was at first experienced in purifying samples of thiodiglycol, which was incorporated in some of the buffers to prevent oxidation of methionine (Moore & Stein, 1951). Even after distillation *in vacuo*, the thiodiglycol gave rise to a yellow colour on heating with the ninhydrin reagent. This colour was found to be produced by an unidentified impurity present in the more volatile fractions. This difficulty was overcome by distilling over the first 30% of the once-distilled thiodiglycol and retaining the remaining 70% left in the distilling flask, for use in the buffers. The purified material was a pale yellow liquid, b.p. 142° at 2 mm. Hg pressure and n_D^{12} 1·5200.

The volumes of standard NaOH and HCl solutions quoted by Moore & Stein (1951) for adjusting certain buffer solutions to pH 5·0 were found to be incorrect. The following volumes were found necessary for neutralization of 1 ml. of buffer: pH 3·42, 0·1 M citrate, 0·12 ml. of N-NaOH; pH 4·25, 0·1 M citrate, 0·063 ml. of N-NaOH; pH 6·7, 0·1 M citrate, 0·042 ml. of 2 N-HCl; pH 6·8, 0·1 M phosphate, 0·09 ml. of 0·5 N-HCl; pH 6·5, 0·2 M citrate, 0·075 ml. of 2 N-HCl.

Glucosamine and galactosamine were separated from each other and from amino acids on a 15 cm. Dowex 50 column (Eastoe, 1954) and were estimated by the ninhydrin method.

RESULTS

Analysis of whole bone tissue

Extraction with cold water. Powdered bone (10 g.) was extracted with cold water by continuous leaching. The bone powder was finally washed and the filtrate and washings made up to 200 ml. The solution showed a small negative optical rotation and had a solids content of 0·98% (dried at 105°) of the weight of bone. The residue, after ashing, was 0·41% of the weight of the bone. No reducing sugars could be detected in the solution. A hydrolysate of the soluble material was examined by paper chromatography and the amino acid pattern was found to be indistinguishable from that of bone collagen. This confirmed the suggestion that the water-soluble organic matter consisted mainly of a gelatin or procollagen type protein or its degradation products (Rogers *et al.* 1952).

Reducing sugars. Hydrolysates of powdered bone were prepared using different concentrations of acid with various heating times at 100°. In none of the hydrolysates did the reducing sugar content exceed 0·2% of the bone tissue. When allowance was made for the interfering effects of amino acids on the method of estimation it was considered that not more than 0·05% of reducing sugar residues were present.

Chemical composition of compact bone. The results of the gravimetric analysis of the air-dried bone powder are shown in Table 1. The organic matter, soluble in hot water, was considered to be mainly degraded gelatin, derived from the collagen. The weight of collagen was assumed to be the weight of

Table 1. *Composition of air-dried compact bone tissue (ox femur diaphysis)*

	% by wt.
Inorganic matter, insoluble in hot water (probably including up to 1% of citrate)	69·66
Inorganic matter, soluble in water	1·25
Collagen	18·64
Mucopolysaccharide-protein complex	0·24
Resistant protein material	1·02
Fat	0·00
Sugars, other than in mucopolysaccharide	0·00
Water (lost below 105°)	8·18
Total	98·99

Table 2. *Inorganic matter in compact bone tissue (ox femur diaphysis)*

	% by wt.
Total inorganic matter (from Table 1)	70·91
Bone, ashed at 450°	70·1
Bone, ashed at 500°	68·7
Bone, ashed at 550°	67·9

the soluble organic matter minus the weight of mucopolysaccharide-protein complex obtained by lime-water extraction. This complex would probably slowly break down and dissolve in hot water.

Almost 99% of the weight of bone was thus accounted for. The remaining 1% was attributed to small accumulated errors in the gravimetric analysis. The difficulty of allowing for the different weights of water retained at 105° by bone powder, the inorganic residue and the organic matter, respectively, is not readily overcome. All of the values in the table were obtained by direct weighing except for the comparatively small weight of resistant protein, which was calculated from the total nitrogen content of the insoluble residue.

The combined weight of the soluble and insoluble inorganic matter (Table 1) agrees well with the values for the residue from bone, ashed at various temperatures (Table 2). The first value quoted is high, due to inclusion of citrate and water retained at 105°. The ash value at 450° probably tends to be high also, owing to the difficulty of burning off carbon at this temperature, while the values for 500 and 550° may be low owing to loss of carbon dioxide from the carbonate.

Total nitrogen content of bone. The air-dried bone, the composition of which is given in Table 1, was found to contain 3·62% N. Assuming that demineralized bone contains 18·1% N, this value corresponds to 20·0% of total organic matter in the bone. This is in good agreement with the value of 19·9% for the total of collagen, resistant protein and mucopolysaccharide complex (Table 1).

Collagen

The total nitrogen content of demineralized bone shavings was found to be 18·10%, which was appreciably lower than the generally accepted value for hide collagen of 18·6% (Bowes & Kenten, 1948). This was at first attributed to decomposition during demineralization, contamination with mucopolysaccharide or both. A number of samples were prepared in which finely powdered bone was rapidly demineralized with sulphurous acid, a comparatively mild treatment, and the mucopolysaccharide was later removed by calcium chloride extraction (see Materials). All of these preparations had a nitrogen content close to 18·26%, only slightly higher than the earlier value. These low values are possibly attributable to the presence of a protein resistant to hot water, which had a nitrogen content of only 14·6%. No method has been found for removing this impurity from collagen, without converting the collagen into gelatin.

Paper chromatograms of bone collagen showed an amino acid composition which could not be distinguished from that of hide collagen. Quantitative studies of the amino acid composition of bone collagen are in progress.

Resistant protein

This residue consisted partly of a stringy elastic mass, resembling 'cartilage tubes' (Le Gros Clark, 1945) and partly of an amorphous powder. The dry, ash-free material contained 14·62% N. This value was probably low compared with that of the main protein constituent of the residue, due to the presence of impurities unavoidably concentrated in a product of a residual nature. The amino acid picture, obtained from the examination of paper chromatograms, was quite unlike those of collagen and elastin (from ligamentum nuchae). There was a closer resemblance to blood albumin, although several slight differences were noted. The protein had a particularly high tyrosine content. The amino acid composition appeared to be closely similar to that reported for the reticular tissue of lymph nodes (Bowes & Kenten, 1949).

As the resistant protein was not homogeneous, no quantitative study of the amino acid composition was made. It is probable that the composition varies with the time of autoclaving, since the resistant protein passes gradually into solution.

Mucopolysaccharide-protein complex

The complex, as isolated, was a white amorphous powder, soluble in dilute alkali and reprecipitated by addition of acid. The alkaline solution was noticeably viscous. The air-dried material contained 9·18% moisture and no ash. The dry material contained C, 46·5; H, 6·5; N, 11·3; S, 1·83; SO_4S, 1·63%. Pure chondroitin sulphate $(C_{14}H_{21}O_{14}NS)_n$, which may be regarded as typical of the sulphur-containing mucopolysaccharides, would contain N, 3·05 and S, 6·97%. The low sulphur content of the isolated

material suggested that only a small part of it could be a sulphated polysaccharide, while the high nitrogen content indicated the possible presence of a protein. Most of the sulphur present was in the form of sulphate, the remaining 0·2% being compatible with the sulphide sulphur of a protein. After 8 hr. hydrolysis with 4N hydrochloric acid at 100°, the average value for the hexosamines present was 9·46%. During the heating, browning of the hydrolysate took place and also some decomposition of the hexosamines. Experiments in which known amounts of glucosamine hydrochloride were added to gelatin before hydrolysis, indicated that some 20% of the hexosamine decomposed. The corrected value for total hexosamines was therefore 12·1%.

An attempted determination of uronic acids failed, owing to the decomposition of a substantial proportion of these sugar derivatives during hydrolysis (4 hr. at 100° with 4N hydrochloric acid). The results obtained for uronic acid liberated and remaining undecomposed under these conditions indicated a minimum content of 3·0%. The true value probably much exceeded this. The estimation of the total reducing sugars present in hydrolysates also failed, owing to interference by amino acids. The results for the hexosamine and uronic acid determinations, like those for the total nitrogen and sulphur, suggested that only a part of the complex was a sulphated polysaccharide.

The sugar residues present in hydrolysates were tentatively identified by paper chromatography. A chromatogram developed with *n*-butyl acetate: acetic acid:water and sprayed first with alkaline acetylacetone and then with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), showed two pink spots of low R_F value, merging into one another, in the positions corresponding to galactosamine (chondrosamine) and glucosamine (chitosamine). A similar chromatogram sprayed with aniline hydrogen phthalate showed three further spots which were identified as mannose, either galactose or glucose and either galacturonic acid or glucuronic acid. In addition there was a faint pink spot of higher R_F value in the position corresponding to the spots for the pentose sugars, xylose and arabinose. Further chromatograms were developed with *n*-butanol:pyridine:water which separated galactose from glucose; only the former sugar was found to be present in the osseomucoid hydrolysate. In addition, it was shown that the pentose present was xylose and not arabinose. The authors at first considered that xylose arose from decarboxylation of glucuronic acid during the acid hydrolysis. It would appear, however, that all reported attempts to demonstrate this reaction have failed. On the other hand, our evidence is not sufficient to prove the existence of xylose, which commonly occurs in plants, in an animal polysaccharide. The sugar residues in the complex were thus provisionally identified as galactosamine, glucosamine, and hexuronic acid, galactose and a small amount of mannose.

One-dimensional amino acid chromatograms were developed with phenol:water and *n*-butanol: acetic acid:water (4:1:5) systems. The protein portion of the complex was found to be entirely free from hydroxyproline and contained a large amount of tyrosine. When compared with a chromatogram of blood albumin (British Drug Houses Ltd.) a marked resemblance was noticed.

A quantitative amino acid analysis was carried out on the mucopolysaccharide-protein complex by the method of Moore & Stein (1951), the results of which are summarized in Table 3. The amino acid composition was quite unlike that of collagen and elastin, being similar to that of the blood plasma proteins, although differing in some details (Tristram, 1953). The amino acid and hexosamine

Table 3. *Amino acid and hexosamine composition of the mucopolysaccharide-protein complex*

	Weight basis (g./100 g. dry, ash-free material)	Molar basis (moles/10^6 g.)
Alanine	3·70	41·5
Glycine	2·65	35·3
Valine	4·50	38·4
Leucine	7·27	55·4
Isoleucine	3·65	27·8
Proline	4·24	36·9
Phenylalanine	2·86	17·3
Tyrosine	1·98	10·9
Tryptophan	—	—
*Serine	3·61	34·4
*Threonine	4·13	34·7
Cystine	1·13	9·4
Methionine	0·84	5·6
Arginine	3·87	19·4
Histidine	2·65	17·1
Lysine	4·26	29·2
Aspartic acid	9·66	72·6
Glutamic acid	11·67	79·4
†Amide N	1·07	76·4
Hydroxyproline	0·00	0·0
Hydroxylysine	0·00	0·0
Glucosamine	1·23	6·8
Galactosamine	7·67	42·8
Methionine (separated as sulphoxide)	0·25	1·7
Total	81·82	616·6
Average residue weight		114·6
Z‡		115·7
% recovery (wt.)		70·7
% recovery (N)		100·5

* The values for serine and threonine have been corrected for decomposition during hydrolysis by multiplying by factors of 100/89·5 and 100/94·7, respectively (Rees, 1946).

† Not included in totals.

‡ Chibnall (1942).

analysis accounted for all the nitrogen and 70 % of the weight of the complex. The remaining 30 % was attributed to the non-nitrogenous sugar residues.

DISCUSSION

The analysis accounted for 99 % of the weight of the bone in terms of the inorganic matter, collagen, mucopolysaccharide fraction and resistant protein. The complete absence from compact bone of fat and carbohydrates, other than the mucopolysaccharide, is significant. This is not in agreement with the work of Rogers (1949), who found 2–4 % of reducing substances in human femora.

The presence of a protein, resisting solution in hot water, in bone confirms the findings of Rogers *et al.* (1952) and is in agreement with the literature results for other collagenous tissues, e.g. hide (Bowes & Kenten, 1949), and dentine (Stack, 1951). These residual proteins are very difficult to characterize because they undergo gradual decomposition during preparation and are also likely to be contaminated with impurities.

In the present study, a hydrolysate of the entire mucopolysaccharide-protein complex, was examined to discover what residues were present and in what quantities. Previous workers have attempted to isolate purified polysaccharides from the complex. The present findings of the occurrence of galactosamine, hexuronic acid and sulphate sulphur are in agreement with the isolation of a polysaccharide, probably chondroitin sulphate, by earlier workers (Hisamura, 1938; Rogers, 1951). The presence of hexosamine and galactose are also consistent with the composition of another polysaccharide, isolated by Hisamura (1938), which contained these residues, together with a high proportion of protein. The residues detected in our study, with the exception of mannose, were identical with those shown to be present by Masamune *et al.* (1951).

The protein constituent of the complex was shown to be neither collagen nor degraded collagen since it had an entirely different amino acid composition. This finding, together with the work of Hisamura (1938), which showed that the protein was quite firmly combined in the fraction containing hexosamine and galactose, suggested that the protein was not an accidental contaminant, precipitated with the polysaccharides but rather that the protein and some of the carbohydrate residues were intimately associated in the intact bone, possibly as a mucoprotein. The simultaneous precipitation of chondroitin sulphate, the mucoprotein containing hexosamine and galactose, and possibly other polysaccharides would thus result in the production of the mucopolysaccharide-protein complex as a mixture of variable composition.

It would appear that the contemporary picture of connective tissue as made up of collagen with chondroitin sulphate and hyaluronic acid as the only mucopolysaccharides is over-simplified. These latter would account for only galactosamine, glucosamine and glucuronic acid residues whereas galactose and mannose also occur in bone. Consden (1953) has reported the occurrence of glucosamine, galactose, mannose and a trace of glucose in subcutaneous connective tissue.

SUMMARY

1. At least 99 % of the weight of compact mammalian bone (ox femur diaphysis) is accounted for by inorganic matter, collagen, a mucopolysaccharide-protein complex and a protein which resists solution in hot water.

2. Compact bone from ox femora contains no fat and no carbohydrate other than that of the mucopolysaccharide complex.

3. No differences have been found between bone collagen and collagens from other tissues.

4. The protein that resists solution in hot water is heterogeneous, one portion resembling cartilage tubes in appearance and the other being an amorphous powder.

5. The mucopolysaccharide-protein complex contains the sugar residues galactosamine, glucosamine, hexuronic acid, galactose and mannose. Xylose, of unknown origin, was also found in the hydrolysate. The whole of the nitrogen content has been accounted for in a quantitative determination of the amino acids and hexosamines. The amino acid composition of the protein is unlike that of collagen, but bears some similarity to the blood serum proteins.

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Animal Fats

3. THE COMPONENT ACIDS OF OSTRICH FAT*

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In continuation of our study of animal fats (Gunstone & Paton, 1953*a*, *b*) we now report the composition of ostrich fat, which has not been examined previously.

EXPERIMENTAL

The fat used in this investigation was obtained from an adult male ostrich (*Struthio camelus*) which had been in captivity in Edinburgh Zoo. Its diet consisted of pasture supplemented with whole maize. In its natural state the ostrich eats small mammals, birds, snakes, lizards and insects, as well as grass, leaves, fruits, berries and seeds. 'A post-mortem revealed a knotted mass of grass filling the proventriculus and gizzard. A varied bacterial flora was isolated from the bone marrow and there was severe congestion of the duodenum and a fatty infiltration of the liver.' (Information kindly supplied by Mr E. C. Appleby.)

The crude fatty tissue (830 g.) was autoclaved at 120° and broken up in a homogenizer before extraction with light petroleum (b.p. 40–60°). The fat (705 g.) was obtained as a white low-melting solid of iodine value 80·4, saponification equivalent 282·8, and free acid 2·1% (as oleic acid), whilst the mixed acids obtained on hydrolysis had iodine value 83·4 and equivalent 273·3.

The mixed acids (197·6 g.) were divided into three fractions by crystallization from methanol (10 ml. per g.) at −40° overnight and by recrystallization of the insoluble portion at −20° from methanol (10 ml. per g.). The results are shown in Table 1.

Each fraction was methylated by treatment with methanol and either HCl at room temperature (*B* and *C*) or conc. H_2SO_4 at the reflux temperature of the solution (*A*) and the resulting esters distilled through an electrically heated and packed column (Towers, Widnes T. 117) under reduced pressure. From the iodine value and saponification equivalent of each fraction and the spectrographic examination, after alkali isomerization, of selected fractions the composition of the mixed acids was calculated by the methods previously described (Gunstone & Paton, 1953*b*). The results are shown in Table 2.

* Paper 2 of this series: Gunstone, F. D. & Paton, R. P. (1953*b*), *Biochem. J.* **54**, 621.

Table 1. *Low-temperature crystallization of fatty acids from ostrich fat*

	Acids			Esters	
Fraction	Wt. (g.)	(%, w/w)	Iodine value	Iodine value	Sap. equiv.
A. Insoluble at −20°	77·5	39·2	21·1	20·3	280·2
B. Soluble at −20°, insoluble at −40°	60·0	30·4	94·4	88·8	292·5
C. Soluble at −40°	60·1	30·4	154·9	146·6	284·2

Table 2. *Component acids of ostrich fat*

All values except in last column are %, w/w, of total.

	Fraction					
	A	*B*	*C*	Total	% (wt.)*	% (mol.)*
Myristic	0·14	0·05	0·68	0·87	0·9	1·0
Palmitic	23·76	0·69	0·26	24·71	24·8	26·3
Stearic	5·93	—	—	5·93	5·9	5·7
Arachidic	0·45	—	—	0·45	0·4	0·4
Tetradecenoic	—	0·13	0·77	0·90	0·9	1·1
Hexadecenoic	0·44	1·86	3·30	5·60	5·6	6·0
Hexadecadienoic	—	—	0·49	0·49	0·5	0·5
Octadecenoic	8·12	24·65	6·88	39·65	39·8	38·3
Octadecadienoic	0·33	2·67	14·03	17·03	17·1	16·6
Octadecatrienoic	—	—	3·83	3·83	3·8	3·8
As eicosenoic†	—	0·31	—	0·31	0·3	0·3
Unsaponifiable	0·03	0·04	0·16	0·23	—	—

* Excluding unsaponifiable material.
† Includes all unsaturated acids higher than C_{18} (average unsaturation −2·1 H).

The following acids were identified in appropriate fractions: palmitic acid (hexadecanoic acid), m.p. 62°; stearic acid (octadecanoic acid), m.p. 68°; hexadecenoic acid as dihydroxypalmitic acid, m.p. 126–127°; oleic acid as dihydroxystearic acid, m.p. 130–131°; linoleic acid as tetrabromostearic acid, m.p. 111–113°; and linolenic acid as hexabromostearic acid, m.p. 181–182°. An attempt to prepare dihydroxymyristic acid from the fraction richest in tetradecenoic acid gave a very small quantity of a product m.p. 118–119°. Bromination of a fraction considered to contain hexadecadienoic acid gave a derivative, m.p. 176–179°, which was insoluble in light petroleum, and which appeared to be hexabromopalmitic acid. (Found: Br 66·0; calc. for $C_{16}H_{26}O_2Br_6$, Br 65·7%.)

RESULTS AND DISCUSSION

Unsaturated C_{16} *acids.* Spectrographic analysis, after alkali isomerization, of a fraction consisting mainly of C_{16} esters indicated an appreciable $E^{1\%}_{1\,cm.}$ value (223·7) at 234 mμ. and a much smaller value (15·0) at 268 mμ. After allowing for the small quantity of polyethenoid C_{18} acids present, these values were 121·8 and 0·5 respectively. The former was taken to indicate the presence of hexadecadienoic acid (12·1%) in this fraction. Attempts to confirm this by the isolation of tetrabromopalmitic acid from a similar fraction containing no C_{18} acids gave only hexabromopalmitic acid. The small amount of 'hexadecadienoic acid' reported in Table 2 should therefore be regarded as an unidentified mixture of unsaturated C_{16} acids. Clément & Meara (1951) have reported the presence of hexadecadienoic and hexadecatrienoic acids in rabbit fat on the basis of spectrographic evidence following alkali isomerization.

Unsaturated C_{18} *acids.* As noted previously (Gunstone & Paton, 1953*b*) the use of spectrographic constants obtained from polyethenoid acids known to be wholly *cis* may not be justified for calculating the composition of animal fats in which the structure of the polyethenoid acids is doubtful. In python fat (Gunstone & Paton, 1953*b*) and crocodile fat (Gunstone & Russell, 1954) the C_{18} polyethenoid acids appear to be essentially linoleic and linolenic acids, but this is less certain in the present case for the following reasons:

(i) Herb & Riemenschneider (1952) suggest that replacement of the potassium hydroxide–glycol (7·5%) by a more concentrated solution (21%) gives larger constants, thereby increasing the sensitivity of this procedure. When a fraction rich in C_{18} unsaturated acids was examined in this way the results differed from those obtained by the standard procedure thus (all values are % (w/w) of fraction):

Glycol reagent (%)	Octadecatrienoic acid	Octadecadienoic acid	Octadecenoic acid	Total
7·5	15·2	55·7	27·3	98·2
21	10·6	59·0	34·7	104·3

(ii) Bromination of a fraction rich in C_{18} unsaturated acids (Markley, 1947) showed the presence of linoleic acid (22%) and linolenic acid (9%), whilst alkali isomerization gave values of 46·4 and 10·8% for octadecadienoic and octadecatrienoic acids respectively. The large difference in the values for linoleic acid and octadecadienoic acid is probably significant and casts further doubt on the homogenity of the octadecadienoic acid.

Table 3. *Component acids of some bird fats*

All values %, w/w, except for iodine values.

	Grey-goose (abdominal)	Emu (subcutaneous)	Light Sussex hen (abdominal)	Ostrich
Iodine value	57·1	65·8	78·5	80·4
Lauric	12·3	—	—	—
Myristic	8·2	0·9	1·2	0·9
Palmitic	20·3	17·5	24·0	24·8
Stearic	5·6	10·1	4·1	5·9
Arachidic	—	0·6	—	0·4
Tetradecenoic	0·6	0·9	—	0·9
Hexadecenoic	2·5	2·1	6·7	6·1
Octadecenoic	41·6	62·2	42·5	39·8
Octadecadienoic	6·6	5·2	20·8	17·1
Octadecatrienoic	—	—	—	3·8
Unsaturated C_{20-22}	2·3	0·5	0·7	0·3

Component acids of bird fats

The ostrich is reported to be the largest living bird and belongs to the subclass Ratitae. There are very few detailed analyses of bird fats and it is of interest to compare the results obtained in this investigation with those quoted by Hilditch, Sime & Maddison (1942). These are summarized in Table 3. Some sea-bird fats, which are rather different in composition (Lovern, 1938), have not been included in this discussion.

In several respects the figures for the grey-goose are rather unusual and as Hilditch *et al.* (1942) suggest this may be due to the presence of coconut oil in the diet. These figures are accordingly neglected in making the following generalizations.

(i) Saturated acids account for 29–32 % of the total. This value, which is surprisingly constant in view of the variation in iodine value, lies between the corresponding value for amphibians and reptiles (20–30 %) and that for rodents (35–40 %).

(ii) The content of palmitic acid is only slightly below the value of 30 ± 3 % (mol.) said to be characteristic of higher land animals (Banks & Hilditch, 1931; Hilditch & Longenecker, 1937), though the value for the emu seems to be somewhat low.

(iii) The lower values for stearic acid parallel those noted for reptiles, amphibians and rodents, and are in marked contrast to the greater values of many land animals. The results for the emu are again anomalous.

(iv) Unsaturated C_{16} acids attain a value (6–7 %) which recurs frequently in bird and rodent fats.

(v) Unsaturated C_{18} acids comprise 60–70 % of the total acids of bird fats. The unsaturation of these fats is controlled largely by the relative amounts of the various C_{18} unsaturated acids, an increase in iodine value being accompanied by an increase in polyethenoid acids and a decrease in the monoethenoid acid content. Winter & Nunn (1953) have reported similar observations in the C_{20} and C_{22} acids in seal oils.

(vi) The amount of C_{20-22} unsaturated acids is small in each case and contrasts with the larger values recorded for amphibian and reptile fats.

The number of bird fats examined is too small to permit of any but tentative conclusions, but the present results confirm that, so far as fat composition is concerned, this class of animals falls between the reptiles and the rodents and is somewhat closer to the latter.

SUMMARY

1. A quantitative study of the component acids of ostrich fat is reported, and the results are correlated with those previously reported for other bird fats.

2. Palmitic (24·8 %), oleic (39·8 %), and octadecadienoic acid (17·1 %) are the major components, whilst myristic, stearic, arachidic, hexadecenoic, polyethenoid C_{16}, octadecatrienoic and higher unsaturated acids are also present.

We wish to thank Mr E. C. Appleby, of the Zoological Park, Edinburgh, for supplying us with the fat used in this investigation and with certain information regarding its source; the Department of Scientific and Industrial Research for a Maintenance Allowance to one of us (W.C.R.); and Miss M. S. Morton who carried out some preliminary investigations in connexion with this problem.

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Animal Fats

4. THE COMPONENT ACIDS OF CROCODILE FAT

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The order Crocodilia comprises the crocodiles proper, alligators, caimans, and gerials. Information on the fat obtained from animals of this group appears to be limited to reports of some characteristics of the oils (Kobayashi, 1922; Luhr, 1932; Nembrot & Cadrobbi, 1936; Bennett, Brown Coomes, Morton & Raymond, 1950). In this paper we report the investigation of samples of fat from two species of crocodile living in different environments.

EXPERIMENTAL

One sample of fat was obtained from an adult Estuarine Crocodile (*Crocodylus porosus*) of about 20 years. This animal had been kept in captivity in Edinburgh Zoo where its diet consisted of whole dead piglets. The crude fatty material (690 g.) after autoclaving at 120° was broken up in a homogenizer and extracted with light petroleum (b.p. 40–60°) giving a semi-solid fat (585 g.). The second sample of fat, obtained through the kindness of the staff of the Colonial Products Advisory Bureau, came from a crocodile (*C. niloticus*) in Tanganyika living (presumably) in its natural state. Some characteristics of these two fats and of the acids obtained from them are set out in Table 1.

The method of analysis was similar to that already described for python (Gunstone & Paton, 1953) and for ostrich fat (Gunstone & Russell, 1954). The mixed acids were separated into three fractions by crystallization at −40 to −45° and at −20°, and each fraction esterified and then distilled. The results are summarized in Tables 2–4.

From both fats palmitic and stearic acid were isolated from appropriate fractions, and the presence of hexadecenoic, oleic, linoleic and linolenic acids was confirmed by the preparation of the usual derivatives. In addition, myristic acid was isolated from *C. niloticus*.

Bromination of fractions rich in polyethenoid C_{18} acids was effected quantitatively (Markley, 1947) and the results compared with those obtained by the alkali isomerization procedure (see Table 5).

Further evidence about the nature of these C_{18} unsaturated acids was obtained in the case of *C. porosus* fat by observing the spectrum after alkali isomerization at 180° for 0·5, 1·0 and 4·5 hr. The values of $E^{1\%}_{1\,cm.}$ at 234 mμ. were 453, 529 and 486 respectively (see Discussion for the significance of these results).

Fractions from *C. niloticus* rich in C_{20} and C_{22} unsaturated acids were also brominated, and the solid products extensively crystallized from a variety of solvents. Two compounds were obtained and these were probably hexabromoeicosanoic acid (decomposes at 255°; found: Br, 61·4; calc. for $C_{20}H_{34}O_2Br_6$, 61·0%), and decabromodocosanoic acid (melts with decomposition at 260°; found: Br, 70·6; calc. for $C_{22}H_{34}O_2Br_{10}$, 70·7%).

DISCUSSION

Unsaturated acids

Evidence of the presence of polyethenoid C_{18} acids is derived only from spectroscopic data, and it is probable that these acids are a complex mixture (cf. discussion of similar acids in ostrich fat, Gunstone & Russell, 1954).

For the C_{18} acids the agreement in the values obtained by bromination and by spectroscopic measurements after alkali isomerization (see Table 5) suggests that the octadecadienoic and octadecatrienoic acids of *C. porosus* are essentially the *all-cis* isomers found in vegetable oils. This conclusion is borne out for the dienoic acid by the fact that maximal absorption was reached after isomerization for 1 hr., whereas Jackson, Paschke, Tolberg, Boyd & Wheeler (1952) report longer times for the *trans-trans* acid (360 min.) and *cis-trans* acids (150 min.). The results obtained in the bromination of *C. niloticus* fractions are less definite, and though linoleic and linolenic acids are present they may be accompanied by isomeric compounds.

The unsaturated acids higher than C_{18} are rather complex. The unsaturated C_{20} acids of *C. porosus* fat appear in fractions *B* and *C*. That portion which is present in fraction *B* must be largely monoethenoid

Table 1. *Characteristics of crocodile fat*

	Fat			Mixed acids	
	Iodine value	Sapon. equiv.	Free acid (as % oleic)	Iodine value	Sapon. equiv.
C. porosus	80·5	278·9	3·4	83·9	267·4
C. niloticus	93·3	283·2	0·4	96·8	271·5

Table 2. *Low-temperature crystallization of crocodile fatty acids*

Fraction	*C. porosus*			*C. niloticus*		
	A	*B*	*C*	*A*	*B*	*C*
Weight (g.)	68·5	58·1	80·0	62·3	37·9	102·4
% (w/w) of total	33·2	28·1	38·7	30·8	18·7	50·5
Iodine value	10·3	85·8	143·5	9·4	89·5	153·1

Table 3. *Component acids of* Crocodylus porosus *fat*

All values %, w/w, except last column.

	Fractions			Total	% (wt.)*	% (mol.)*
	A	*B*	*C*			
Lauric	—	—	0·44	0·44	0·4	0·6
Myristic	0·80	0·83	1·22	2·85	2·9	3·4
Palmitic	23·58	2·51	0·43	26·52	26·6	28·1
Stearic	4·75	—	—	4·75	4·8	4·5
Arachidic	0·56	—	—	0·56	0·6	0·5
Dodecenoic	—	—	0·17	0·17	0·2	0·2
Tetradecenoic	—	0·18	0·90	1·08	1·1	1·3
Hexadecenoic	0·17	1·17	4·87	6·21	6·2	6·6
Hexadecadienoic	—	—	0·32	0·32	0·3	0·3
Octadecenoic	3·06	19·24	11·15	33·45	33·5	32·2
Octadecadienoic	0·25	1·43	15·29	16·97	17·0	16·4
Octadecatrienoic	—	—	2·61	2·61	2·6	2·6
As eicosenoic†	—	2·67	1·08	3·75	3·8	3·3
Unsaponifiable	0·03	0·07	0·22	0·32	—	—

* Excluding unsaponifiable material. † Average unsaturation – 3·5H.

Table 4. *Component acids of* Crocodylus niloticus *fat*

	Fractions			Total	% (wt.)*	% (mol.)*
	A	*B*	*C*			
Myristic	0·79	1·14	1·98	3·91	3·9	4·7
Palmitic	22·25	1·32	0·35	23·92	24·0	25·6
Stearic	3·40	—	—	3·40	3·4	3·3
Arachidic	1·34	—	—	1·34	1·3	1·2
Tetradecenoic	—	0·27	0·71	0·98	1·0	1·2
Hexadecenoic	0·29	1·36	12·68	14·33	14·4	15·4
Hexadecadienoic	—	—	0·32	0·32	0·3	0·3
Hexadecatrienoic	—	—	0·26	0·26	0·3	0·3
Octadecenoic	2·60	12·91	15·13	30·64	30·8	29·7
Octadecadienoic	0·12	0·65	5·67	6·44	6·5	6·3
Octadecatrienoic	—	—	3·05	3·05	3·1	3·0
Eicosenoic†	—	1·00	5·72	6·72	6·8	5·7
Docosenoic‡	—	—	4·22	4·22	4·2	3·3
Unsaponifiable	0·01	0·05	0·41	0·47	—	—

* Excluding unsaponifiable material.
† Average unsaturation – 5·8H.
‡ Average unsaturation – 7·9H.

Table 5. *Polyethenoid* C_{18} *acids*

Values are %, w/w, of fractions containing only C_{18} acids.

	Bromination		Alkali isomerization	
	Diene	Triene	Diene	Triene
C. porosus	48·7	12·9	44·4	14·3
C. niloticus	16·9	17·9	12·8	23·8

Table 6. *Component acids of some amphibian and reptile fats*

All values %, w/w. Values are taken from Hilditch (1947) apart from the results for turtle (ii) (Giral & Marquez, 1948), python (Gunstone & Paton, 1953) and crocodile (present work). Values in parentheses are mean unsaturations.

			Turtle		Crocodile		Lizard		
	Frog	Tortoise	(i)	(ii)	(i)	(ii)	(i)	(ii)	Python
Lauric	—	—	13·3*	—	0·4	—	—	—	—
Myristic	4	1	10·6	6·6	2·9	3·9	4	4	1·3
Palmitic	11	14	17·0	21·8	26·6	24·0	18	29	19·7
Stearic	3	4	4·1	15·5	4·8	3·4	7	10	10·8
Arachidic	—	—	—	1·9	0·6	1·3	—	—	1·2
Tetradecenoic	—	—	1·3	3·5	1·1†	1·0	—	—	0·5
Hexadecenoic	15	9	7·8	18·0	6·5	15·0	10	12	3·9
Octadecenoic	52	65	39·6	31·4	33·5	30·8	56	40	47·0
Octadecadienoic					17·0	6·5			10·7
Octadecatrienoic	(−2·5H)	(−2·4H)	(−2·2H)	(−3·7H)	2·6	3·1	(−2·4H)	(−2·7H)	0·8
'Eicosenoic'	15	7	6·1	1·3	3·8	6·8	5	5	4·1
'Docosenoic'	(−6H)	(−4H)	(−6·3H)	(−8·6H)	—	4·2	(−5H)	(−5·5H)	—

* Also 0·2% decanoic acid. † Also 0·2% dodecenoic acid.

in view of its low iodine value (approx. 90), its small absorption at 234 mμ. (approx. 40) after alkali isomerization, and the fact that saturated acids should not be present in this fraction. The homologous acids in fraction *C* are more unsaturated, and since they show appreciable absorption, after alkali isomerization, at 234, 268, 300 and 315 mμ. they must contain a tetraethenoid acid, possibly accompanied by less unsaturated C_{20} acids. The higher unsaturated acids of *C. niloticus* are present in greater amounts, and include C_{22} as well as C_{20} acids. Both occur almost entirely in fraction *C* and are highly unsaturated (iodine value 220–300). Alkali isomerization of the C_{20} fraction gives absorption maxima at 234, 268, 300, 315 and 346 mμ., showing that pentaethenoid acids are present, whilst bromination experiments indicated that eicosatrienoic and docosapentaenoic acids occur in the C_{20} and C_{22} fractions.

Depot fat of amphibians and reptiles

Klenk (1933) and Klenk, Ditt & Diebold (1935) have drawn attention to the fact that the fatty acid composition of certain amphibian and reptile fats studied were intermediate in nature between the fats of sea and of land animals. Results obtained later have not made it necessary to contradict this, nor do the present results on crocodile fat. The quantitative differences in the values recorded in Table 6 make it difficult to point to any generalization, though it is apparent that in all cases hexadecenoic acid makes an appreciable contribution (4–15%) whilst the C_{20-22} unsaturated acids are present in similar amounts (4–15%). It can be seen further that in any fat the amounts of these two groups of acids are very similar, small amounts of hexadecenoic acid being associated with small amounts of the C_{20-22} unsaturated acids and vice versa.

Another factor which makes it difficult to draw comparisons lies in the differences in the two analyses of lizard fat, turtle fat, and of crocodile fat. In the present case the two samples are from different species and, in addition, one had been kept in captivity for several years whilst the other had lived in its natural state. In comparing the compositions of the two crocodile fats two points are worthy of note: (i) despite the difference in iodine value between these two samples there is little difference in the content of saturated acids, all the differences being in the relative amounts of the unsaturated acids; and (ii) the more unsaturated fat, obtained from *C. niloticus* living in its natural state, contained a greater proportion of C_{16} and of C_{20-22} unsaturated acids, whilst the *C. porosus* sample contained more octadecadienoic acid which was more certainly linoleic acid than in the former case. These differences may reflect differences in diet, since the captive animal was fed on whole dead piglets, whilst crocodiles in their natural state are reported to eat mainly fish. Fish fats are known to contain high proportions of hexadecenoic and of C_{20-22} unsaturated acids.

Bennett *et al.* (1950) have suggested that crocodile oil might find a use alongside turtle oil in cosmetic preparations. Comparison of our results for *C. niloticus* (this animal was in its natural state and belongs to the species potentially available) with those given by Giral & Marquez (1948) for turtle oil shows a marked similarity apart from the greater proportion of unsaturated C_{20-22} acids in the crocodile oil and somewhat less stearic acid.

SUMMARY

1. The component acids of two samples of crocodile fat, *Crocodylus porosus* and *C. niloticus* are reported.

2. Each oil contained similar quantities of saturated acids (33–35%), mainly palmitic (24–27%), but differed in the proportions of the various unsaturated acids. These differences are particularly evident in the content of hexadecenoic acid, octadecadienoic acid, and the unsaturated C_{20-22} acids.

We thank Mr E. C. Appleby, of the Zoological Park, Edinburgh, for the sample of *C. porosus* fat; Dr W. D. Raymond, O.B.E., of the Colonial Products Advisory Bureau (Plant and Animal), for the sample of *C. niloticus* fat; and the Department of Scientific and Industrial Research for a maintenance allowance to one of us (W.C.R.).

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The Sulphatase of Ox Liver

3. FURTHER OBSERVATIONS ON SULPHATASE *B* AND AN INVESTIGATION OF THE ORIGIN OF FRACTIONS *A* AND *B*

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The previous communications of this series have described the occurrence of two fractions exhibiting sulphatase activity in an aqueous extract of an acetone powder of ox liver, and the purification of one of these fractions, sulphatase *A* (Roy, 1953*a*, *b*). The present paper describes the further purification of the second fraction, sulphatase *B*, as a preliminary to the study of the relationship between the two fractions, more especially the possibility of artifact formation during their preparation. This is of particular interest as Dodgson, Spencer & Thomas (1953) have not detected two fractions in their preliminary study of rat liver sulphatase, although unpublished work from this laboratory has shown that two fractions, comparable to fractions *A* and *B* of ox liver, can be isolated from an acetone powder of rat liver by the methods already described (Roy, 1953*a*).

EXPERIMENTAL

Preparation of the enzyme

The general methods of preparing the acetone-powder extract and of fractional precipitation with acetone or $(NH_4)_2SO_4$ were as described previously (Roy, 1953*a*, *b*). Protein estimations were kindly performed by Dr L. M. H. Kerr using a slight modification of the Folin phenol-method of Lowry, Rosenbrough, Farr & Randall (1951). In what follows, one sulphatase *B* unit (s.u.) is defined as the amount of enzyme which will liberate 1 μg. nitrocatechol under the standard conditions described below.

Stage B. This was obtained by the precipitation of 800 ml. aqueous extract with 43% (v/v) acetone in phosphate buffer, pH 7, at $-9°$ as described by Roy (1953*a*). The precipitate so obtained was dissolved in water and dialysed overnight against running tap water at room temperature, giving 400 ml. of sulphatase *B* (700 000 s.u., 25 s.u./mg. protein).

Stage B-1. To 400 ml. sulphatase *B* were added 26 ml. 0·5 M sodium acetate, pH 6·5, and 4·5 ml. 0·3 M-$CaCl_2$. The enzyme was then precipitated by the addition of 220 ml. acetone (34%, v/v, final concentration), the temperature being lowered to $-9°$ during the process. After equilibration at that temperature the precipitate was centrifuged off, dissolved in 100 ml. water and dialysed as above. A copious inactive precipitate was separated, giving 250 ml. sulphatase *B* (stage *B*-1; 500 000 s.u., 36 s.u./mg. protein).

Stage B-2. 250 ml. *B*-1 were made 0·3 saturated with respect to $(NH_4)_2SO_4$ by the slow addition of 55 g. solid $(NH_4)_2SO_4$. After standing a few hours the precipitate was centrifuged off and discarded. The supernatant was made 0·5 saturated with respect to $(NH_4)_2SO_4$ by the addition of the calculated amount of solid and after equilibration the active precipitate was centrifuged off, dissolved in water and dialysed overnight. This $(NH_4)_2SO_4$ fractionation was then repeated on the dialysate, giving a clear solution of sulphatase *B*-2 (200 000 s.u., 190 s.u./mg. protein).

Estimation of sulphatase B activity

The substrate used was dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) and the enzymic activity was followed by the colorimetric estimation of the liberated 4-nitrocatechol (Robinson, Smith, Spencer & Williams, 1952; Roy, 1953*a*). A buffered substrate solution was prepared by dissolving 250 mg. nitrocatechol sulphate (dihydrate) in approximately 5 ml. hot water; after adding 0·58 ml. N-HCl and 3 ml. 0·5M acetate buffer, pH 5·7, the volume was made up to 9 ml. with water, giving a 0·08M solution of nitrocatechol sulphate, pH 5·7. This solution was stored at 37°, as below this temperature crystallization occurred. For assay, 0·2 ml. enzyme solution was mixed with 0·6 ml. buffered substrate, both solutions being at 37°, and the reaction mixture incubated 1 hr. at that temperature. The final substrate concentration was therefore 0·06M nitrocatechol sulphate in 0·15M acetate buffer, pH 5·7. After incubation the solution was deproteinized with 3 ml. 2% (w/v) phosphotungstic acid in 0·1N-HCl and the liberated nitrocatechol estimated colorimetrically by means of the red colour developed in an alkaline quinol solution. Assays were run in duplicate along with appropriate blanks. Under the above conditions the reaction velocity was directly proportional to the enzyme concentration and the degree of hydrolysis of the substrate to the time of incubation for periods of up to 2 hr. If the protein-free supernatant from the phosphotungstic acid precipitation was left for some time before making alkaline, a copious white precipitate slowly separated. No attempt was made to remove this precipitate as it immediately dissolved in the alkaline quinol. The precipitation was apparently due to a high concentration of electrolytes.

Electrophoresis on paper

Paper electrophoresis was carried out in an apparatus developed from that of Latner (1952). Approximately 0·07 ml. of the solution to be analysed (containing approximately 4 mg. protein) was lined on a strip (50 × 6 cm.) of Whatman no. 100 paper and run for 16 hr. at 200 v in a veronal–acetate–HCl buffer of suitable pH, usually pH 7·1. The various components were localized by cutting the paper into 1 cm. strips and assaying portions of these strips (usually 1 × 3 cm.) as described below.

Total protein. The strip was immersed in 1·0 ml. diluted Folin and Ciocalteu reagent (British Drug Houses reagent diluted 1 in 10 with water) and allowed to stand for 15 min.; 1 ml. N-Na_2CO_3 was then added and the tubes were incubated 15 min. at 37° to develop the colour, the intensity of which was indicative of the amount of protein present.

Sulphatases A and B. These were localized by incubating the strips for a suitable time (normally 1 hr.) at 37° in 0·8 ml. acetate-buffered nitrocatechol sulphate, 0·003M, pH 5·0, for sulphatase *A*, and 0·06M, pH 5·7, for sulphatase *B*. After incubation, the reaction was stopped with phosphotungstic acid and the colour developed with alkaline quinol as usual.

Acid phosphatase. The strips were incubated for 1 hr. at 37° in 0·8 ml. 0·0025M disodium *p*-nitrophenyl phosphate in 0·15M acetate buffer, pH 5·0. Phosphatase activity was detected by the addition of 8 ml. 0·6N-Na_2CO_3 to develop a yellow colour in these tubes containing liberated *p*-nitrophenol.

Esterase. This was detected by the method of Huggins & Lapides (1947) by incubating the strips in 2 ml. 0·06M phosphate buffer, pH 7, 2 ml. 0·066M *p*-nitrophenyl acetate, and 6 ml. water at 20°. In the presence of an esterase a yellow colour developed.

All these methods could be made semi-quantitative by reading the intensity of colours in the Spekker Absorptiometer after removing the filter paper by centrifuging.

RESULTS

Properties of sulphatase B

Effect of variations in substrate concentration. The effect of variations in substrate concentration on the reaction velocity is shown in Fig. 1. It is obvious that the maximum velocity has not been reached even at a substrate concentration of 0·06M nitrocatechol sulphate. The results give a good fit of the Lineweaver & Burke (1934) equations, and a number of experiments gave values of K_m between 0·06M and 0·08M nitrocatechol sulphate. It is clear that the optimum substrate concentration must be exceptionally high, in the region of 0·2M nitrocatechol sulphate, a region in which it is impossible to work owing to the limited solubility of the substrate.

Effect of pH. Buffered substrate solutions were made up as described above using acetate buffers of varying pH and the appropriate amount of N-HCl, as determined from the titration curve of nitro-

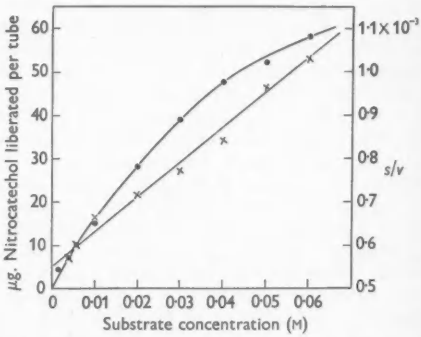

Fig. 1. Effect of varying substrate concentration on the reaction velocity. Incubated 1 hr. at 37°, with varying concentrations of nitrocatechol sulphate in 0·15M acetate buffer, pH 5·7. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution; ●—●, plot of reaction velocity against substrate concentration; ×—×, the above data plotted according to the equation derived by Lineweaver & Burke (1934)

$$\frac{s}{v}=\frac{1}{V}s+\frac{K_m}{V},$$

where s is the substrate concentration, v and V the observed and maximum velocities respectively, and K_m the Michaelis constant.

catechol sulphate. As Fig. 2 shows, the optimum pH was 5·7 under such conditions. The use of acetate buffers of such high pH values is justified by the very strong buffering action of nitrocatechol sulphate in that region, the phenolic group having a pK of approximately 6·4.

When citrate buffers were used in place of acetate, a considerable inhibition occurred, but the position of the optimum was unchanged at pH 5·7.

Inhibition studies. These were normally carried out by dissolving the inhibitor to the required concentration in 0·17 M acetate buffer, pH 5·7, adjusting the pH as necessary. For assay, 0·1 ml. of the buffered inhibitor was mixed with 0·5 ml. buffered substrate and 0·2 ml. enzyme added, so that the final substrate concentration was 0·05 M nitrocatechol sulphate.

Like sulphatase A, sulphatase B was unaffected by 0·05 M-NaCl or KCl, and by 0·01 M-KCN. It was inhibited to 25% by $2{\cdot}5 \times 10^{-2}$ M-Na_2SO_4 and to 40% by 3×10^{-4} M-Na_2SO_3. Investigation of the inhibition by these substances suggested that it was non-competitive with values of K_i of approximately 7×10^{-2} M-Na_2SO_4 and 5×10^{-4} M-Na_2SO_3, respectively. Too much stress must not be laid on the non-competitive nature of the inhibition as the range of substrate concentrations studied was of necessity small. A number of sulphuric acid esters were studied as possible inhibitors of sulphatase B. The results are not reported as they showed nothing of interest, the inhibition in all cases being rather less than the inhibition of sulphatase A by the same concentration of any given ester (Roy, 1953*b*). Sulphatase B was not inhibited by a final concentration of 1 mg./ml. of the methiodide of *m*-dimethylaminophenyl methyl carbamate, a potent inhibitor of liver esterase (Stedman & Stedman, 1931). Sulphatase A is likewise uninhibited by this urethane.

Activation energy. The apparent activation energy for the hydrolysis of nitrocatechol sulphate by sulphatase B was determined at pH 5·7 and a substrate concentration of 0·06 M nitrocatechol sulphate by determining the enzymic activity at various temperatures between 20 and 40°. From the results, shown in Fig. 3, the activation energy was calculated (Arrhenius, 1889) to be 13000 cal./mole. As pointed out by Gibson (1953), on theoretical grounds, the true activation energy can only be determined at substrate concentrations approaching the optimum; at lower concentrations the determined value is greater than the true value by a variable amount, so that the above value of 13000 cal./mole is a maximal figure. It should be noted, however, that Mills, Paul & Smith (1953) claimed that the determined activation energy for the hydrolysis of phenyl glucuronide by β-glucuronidase was less than the true value at substrate concentrations below the optimum.

Electrophoretic investigation of sulphatase B. Preparations of sulphatase B-2 had been shown in preliminary work (unpublished observations) to exhibit considerable acid phosphatase and esterase activity. It has in the past been implied that sulphatase might be identical with a phosphatase or an esterase (Hommerberg, 1931) and it was therefore decided to attempt the separation of the three enzymes electrophoretically.

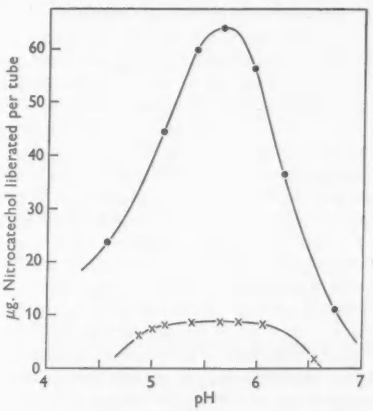

Fig. 2. Effect of pH on reaction velocity. Incubated 1 hr. at 37° in 0·15 M acetate or citrate buffers. Substrate concentration 0·06 M nitrocatechol sulphate. Final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution; ●—●, acetate buffer; ×—×, citrate buffer.

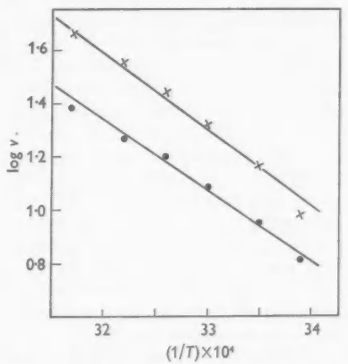

Fig. 3. Plot of log reaction velocity (log v) against the reciprocal of absolute temperature ($1/T$) for two concentrations of enzyme. Substrate concentration 0·06 M nitrocatechol sulphate, pH 5·7 in 0·15 M acetate buffers, final volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme. Incubated 1 hr.

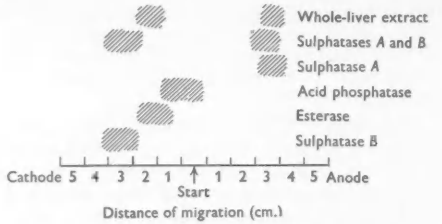

Fig. 4. Typical results of electrophoretic investigations of sulphatase preparations. Run 16 hr. at 200 v; pH 7·1 in veronal buffer.

The results of typical experiments are indicated in Fig. 4, which conclusively shows the non-identity of the three enzymes. For comparison, the migration under similar conditions of sulphatase *A* is shown, and it is obvious that this enzyme is also distinct. It was also possible to separate by electrophoresis the two types of sulphatase activity from a mixture of sulphatases *A* and *B*.

Origin of sulphatases A and B

The low affinity of sulphatase *B* for its substrate suggested the possibility that it might be an artifact produced by the acetone treatments utilized in its preparation. In fact, the possibility could not be excluded that both sulphatases were artifacts. This problem was investigated by a study of the kinetics of various types of enzyme preparation and by electrophoresis.

Study of the acetone-powder extract. A sample of an unfractionated extract of an acetone powder was fractionated with acetone as previously described (Roy, 1953*a*) and the two fractions so obtained were combined after dialysis overnight. A similar sample of unfractionated extract was dialysed without preliminary treatment, and the volumes of both dialysed samples were adjusted so that the enzyme concentrations should have been the same in both. The effect of variations in pH on the enzymic activity of both samples was determined in 0·15 M acetate buffers and at substrate concentrations of 0·003 M and 0·06 M nitrocatechol sulphate. Fig. 5 shows the results of such an experiment and it can be seen that no significant change in the nature of the pH effects was brought about by the acetone treatment. It was therefore concluded that both sulphatases *A* and *B* existed in an aqueous extract of an ox-liver acetone powder.

Study of liver dispersions. A 5% dispersion of fresh ox liver in water, final pH 6·7, was prepared by treatment for 30 sec. in an Atomix Blender. A sample of the dispersion was brought to pH 4·7 with dilute acetic acid, stood for 10 min. at 37°, and the pH readjusted to 6·7 with dilute Na_2CO_3, giving the 'acetate-treated' enzyme. A similar sample was

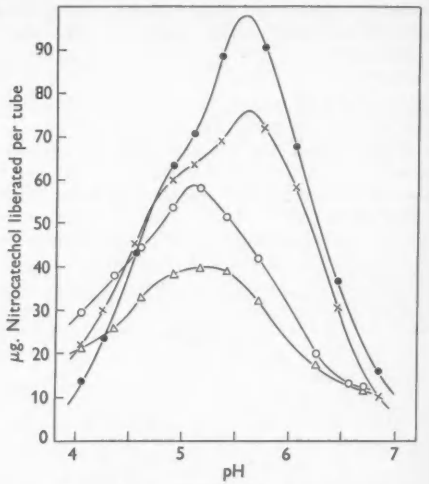

Fig. 5. Effect of pH on reaction velocity of an unfractionated extract of an acetone powder of ox liver (●—● and ○—○, at substrate concentrations of 0·06 M and 0·003 M nitrocatechol sulphate, respectively) and of the combined fractions *A* and *B* isolated therefrom (×—× and △—△, at substrate concentrations of 0·06 M and 0·003 M nitrocatechol sulphate, respectively). Assay in 0·15 M acetate buffers, general conditions as in Fig. 2.

centrifuged for 10 min. at 600 *g* after incubation as above giving two fractions, the supernatant, ('acetate-soluble') which was made up to the original volume of the sample, and the residue ('acetate-insoluble'), which was suspended in water and also made up to the original volume. The pH of both these fractions was also readjusted to 6·7. The pH/activity curves of these four fractions were then determined in 0·15 M acetate buffers and a substrate concentration of 0·03 M nitrocatechol sulphate. Fig. 6 shows the results of typical experiments. It is obvious that there is no significant difference in the shape of the pH curves of the untreated and acetate-treated enzymes, and that the curves of the acetate-soluble and acetate-insoluble fractions are both very different in nature from those of the first two preparations. Moreover, the summation of the pH curves of the acetate-soluble and acetate-insoluble fractions gave a curve almost identical with that of the acetate-treated enzyme. It should be noted that the recovery of nitrocatechol from liver dispersions prepared as described above is quantitative at all pH values studied.

Fig. 7 shows the effect of variations in substrate concentration on all four fractions. The pH was 5·5 in 0·15 M acetate buffer. Again it is obvious that acetate treatment alone causes no change in the properties of the enzyme, and that the acetate-

soluble and acetate-insoluble fractions have different properties from the whole-liver dispersion.

The above experiments suggested that both sulphatases *A* and *B* were present in the liver dispersion, and that the acetate-soluble fraction was predominantly sulphatase *B*, while the acetate-insoluble fraction was probably a mixture of approximately equivalent amounts of sulphatases *A* and *B*.

Electrophoretic investigation of whole-liver extract. An approximtely 20 % dispersion of fresh ox liver in water was prepared as above and centrifuged at 30000 *g* for 1 hr. at 0°. The clear red supernatant, which contained approximately 75 % of the sulphatase activity, was separated and 0·1 ml. portions were taken for electrophoresis as described above. Sulphatase activities corresponding to sulphatase *A* and sulphatase *B* could clearly be detected in such an extract, as indicated in Fig. 4, although the latter activity appeared to move more slowly than purified sulphatase *B* under comparable conditions. As the ox liver was of necessity only available about 1 hr. after the death of the animal, post-mortem changes could not be ruled out and it seemed advisable to repeat the experiment using rat liver, which could be worked up immediately on killing the animal. Again both sulphatases were detected, although there appeared to be relatively less of sulphatase *A* than in ox liver. A similar pattern was also obtained after homogenizing the rat liver in a glass homogenizer (Potter & Elvehjem, 1936) as described by Dodgson *et al.* (1953).

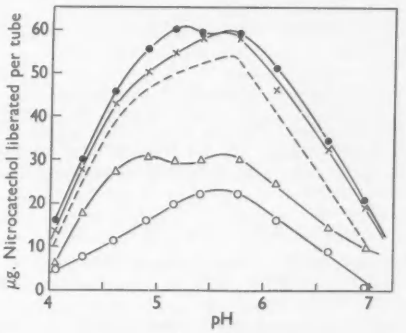

Fig. 6. Effect of pH on reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments; for details of which see text. Assay in 0·15 M acetate buffers, substrate concentration 0·03 M nitrocatechol sulphate; general conditions as in Fig. 2; ●—●, whole dispersion of ox liver; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'; – – –, summation of 'acetate-soluble' and 'acetate-insoluble' curves.

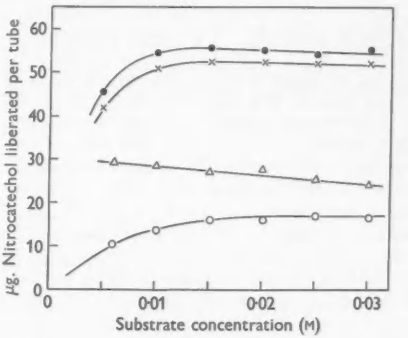

Fig. 7. Effect of varying substrate concentration on the reaction velocity of sulphatase in a dispersion of ox liver before and after various treatments. Assay in 0·15 M acetate buffer, pH 5·5, general conditions as in Fig. 1; ●—●, whole dispersion; ×—×, 'acetate-treated'; △—△, 'acetate-insoluble'; ○—○, 'acetate-soluble'.

DISCUSSION

Little need be said of the properties of sulphatase *B*, the only point of interest being its low affinity for nitrocatechol sulphate, K_m being approximately 0·07 M. This low affinity of an enzyme for its substrate is uncommon, the only obvious counterpart being the low affinity of certain esterases for the simpler esters. One conclusion might be that the sulphatase activity is a secondary action exhibited by an as yet unidentified hydrolase, although the activation energy of 13 000 cal./mole for the hydrolysis of nitrocatechol sulphate by sulphatase *B* is not very different from the corresponding figure for sulphatase *A*, and is very much less than that for the acid hydrolysis of aryl sulphuric acids, which is in the region of 23 000–27 000 cal./mole (Burkhardt, Ford & Singleton, 1936). This could be taken to indicate that a true sulphatase is involved. Table 1 summarizes some properties of sulphatases *A* and *B*.

More important is the question whether or not sulphatases *A* and *B* are present in liver as such. The kinetic studies described above lead to the conclusion that both enzymes are actual constituents of liver tissue, and this view is strongly supported by the results of the electrophoretic studies. This

Table 1. *Comparison of sulphatases A and B*

In both cases the substrate was nitrocatechol sulphate.

	Sulphatase *A*	Sulphatase *B*
pH optimum (in acetate)	4·9	5·7
Optimum substrate concn.	0·003 M	>0·10 M
K_m	8×10^{-4} M	7×10^{-2} M
$K_i\ SO_4^{2-}$	7×10^{-4} M	7×10^{-2} M
$K_i\ SO_3^{2-}$	2×10^{-6} M	5×10^{-4} M

contrasts with the findings of Dodgson *et al.* (1953) who did not detect two enzyme fractions in their study of rat liver sulphatase, although both sulphatases *A* and *B*, isolated from ox liver, will hydrolyse potassium *p*-acetylphenyl sulphate, the substrate used by these workers. It is possible, however, that the method used by Dodgson *et al.* (1953) would not show the presence of two enzymes. Simple kinetic studies on a whole-liver dispersion would not necessarily show that two enzymes were present unless these studies were designed for that purpose. Investigation of the effect of variations of pH and substrate concentration on the sulphatase activity of whole-liver preparations could well lead to the conclusion that only one enzyme was present as such curves are often remarkably smooth, although they are the resultants of two components, corresponding to sulphatases *A* and *B* respectively. Consideration of the pH curve published by Dodgson *et al.* (1953) shows that their results are not inconsistent with the view that two enzymes are present, as the curve shows a flattening in the region of pH 6·5–7·2, analogous to a flattening between pH 5·0 and 5·7 obtained on many occasions in the present study of whole liver dispersions.

The differing pH optima in the two sets of results are not difficult to explain if it be postulated that the formation of the enzyme–substrate complex is due to, or assisted by, electrostatic attraction between the $—SO_4^-$ group of the substrate and a positively charged active centre on the enzyme molecule. At pH 7, the phenolic group of nitrocatechol sulphate is ionized and so might well compete with the $—SO_4^-$ group for the active centre: below pH 5·5 the phenolic group is not appreciably ionized and so could not compete with the $—SO_4^-$ grouping. On this basis it would be expected that the optimum pH for the hydrolysis of nitrocatechol sulphate would be lower than that for aryl sulphates not containing a second ionizable group. This is in agreement with the results reported above.

The most important conclusion is that results of studies of the distribution of sulphatase in various tissues must be accepted with considerable caution until these tissues have been studied with regard to their content of sulphatases *A* and *B*.

SUMMARY

1. A method is given for the partial purification of sulphatase *B* and the properties of the enzyme are described.

2. Sulphatases *A* and *B* have been shown by electrophoretic studies to be distinct from esterase and from acid phosphatase.

3. It has been shown that sulphatases *A* and *B* exist in an aqueous extract of fresh liver both from the ox and from the rat.

4. The difference between the above findings and those of other workers are discussed, and the need for caution in the interpretation of earlier results is stressed.

The author is deeply indebted to Drs Dodgson and Spencer for their co-operation in kindly checking the activity of sulphatases *A* and *B* for the hydrolysis of potassium *p*-acetylphenyl sulphate. He also wishes to thank Dr Edgar Stedman, F.R.S., for a generous gift of the esterase inhibitor.

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The Interaction of Proteins with Silicic Acid

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Although silicon is a constituent of normal animal tissues, silicic acid formed by the dissolution of inhaled quartz particles can induce the formation of pathological lesions in which the normal tissue is replaced by fibrotic tissue arranged concentrically around the silica particles. The mechanism of the change which constitutes the disease, silicosis, is unknown. Schulman & Rideal (1937) demonstrated the interaction of a protein with silicic acid by injecting the acid beneath a monolayer of gliadin at pH 6·2. The silicic acid was adsorbed to the protein layer and after 12 hr. produced a film which could not be penetrated or dispersed by sodium oleate.

This communication describes a more extensive study designed to investigate the conditions under which proteins react with silicic acid and to determine which of their functional groups are responsible for the interaction. Two methods of study were used. Protein monolayers were spread on silicic acid solutions and the characteristics of the films were compared with monolayers on subsolutions which contained no silicic acid. Silicic acid and protein solutions were also mixed in bulk and their interaction was followed turbidimetrically. The two proteins examined, bovine serum albumin and methaemoglobin, were chosen because of the considerable difference between their isoelectric points.

The properties of gliadin films on silicic acid substrates indicate that the interaction involves two processes: (*a*) the adsorption of silicic acid to the protein, and (*b*) the polymerization of the silicic acid. When silicic acid, in the form of the monomer or low polymers, is adsorbed beneath a protein film, polymerization between molecules adsorbed on adjacent protein sites follows and results in the formation of a complete network underneath the protein film, which is thereby solidified. If, however, the protein is spread on a solution containing silicic acid already in the form of high polymers and adsorption occurs, the protein is anchored by the silicic acid and is prevented from spreading completely to a monolayer. If interaction takes place, the area to which the protein will spread may be considerably decreased.

The rate of polymerization of silicic acid depends markedly on pH and, except in the region pH 5·5–6, it is slow. To simplify the interpretation of our measurements and to allow a study of protein-silicic acid interaction without the complicating polymerization reaction, the silicic acid sols were pre-polymerized at pH 6 before use as subsolutions.

A parallel study of the interaction of monolayers of a carboxylic acid, an alcohol and an amine allows some deductions to be made as to which groups of the protein are likely to interact with silicic acid. The influence of pH on the formation of silicic acid-protein complexes is shown most clearly by turbidimetric measurements on bulk solutions.

MATERIALS

Silicic acid. A sample of pure vitreous silica, kindly supplied by the Thermal Syndicate Ltd., was crushed, mixed with 4 times its weight of Na_2CO_3 and fused in a platinum crucible. The melt was dissolved in distilled water. The concentration of the solution was adjusted to about 0·15 M and the solution was brought to pH 6 (glass electrode) by titration with 2 N-HCl. Unless stated otherwise, all silicic acid solutions were first polymerized at pH 6 and at a concentration of 0·1 M for 40 min. before bringing to the required concentration and pH. (Concentrations have been expressed throughout as molarities with respect to $Si(OH)_4$.) Sols of silicic acid are polydisperse and their composition cannot be determined; for a discussion on the state of these sols see the Discussions of the Faraday Society on Colloidal Electrolytes (1935).

Methaemoglobin. This was prepared from human erythrocytes which were washed five times with isotonic saline then lysed with distilled water. The haemoglobin content was estimated and a 20% molar excess of 0·7 M-$K_3Fe(CN)_6$ added.

Poly-L-lysine. The sample was kindly supplied by Dr C. H. Bamford of Courtaulds Ltd., Maidenhead. It was prepared by the Leuchs reaction, as described by Frankel, Grosfeld & Katchalski (1948), and it had a degree of polymerization of 30.

Octadecan-1-ol (m.p. 57°). This was supplied by British Drug Houses Ltd. Its purity was checked by following its force/area curve when spread on 0·01 M-NaCl. The curve corresponded to that obtained by Adam & Dyer (1924).

Bovine serum albumin. A crystalline preparation was supplied by Armour Biochemicals Ltd.

Heptadecylamine hydrochloride was kindly prepared by Dr C. L. Leese from stearic acid by the Schmidt reaction (Briggs, De Ath & Ellis, 1942). The sulphate was converted into the free base by the addition of NaOH. The base was extracted with petroleum ether. Heptadecylamine hydrochloride, m.p. 168°, was precipitated from the solution by dry HCl.

Spreading solvents were kept over activated charcoal for several hours before being filtered and redistilled to remove surface-active impurities.

Water, after distillation, was passed through a mixed-bed resin (Permutit Biodeminerolit) ion-exchange column in a Polythene tube. Its conductivity was less than 2×10^{-7} Ω^{-1} cm.$^{-1}$. All other reagents were of A.R. grade.

EXPERIMENTAL AND RESULTS

Monolayers were studied on a standard Langmuir-Adam trough. The trough itself was cut from block Polythene, and was waxed. The whole apparatus was encased to exclude dust and to provide thermal insulation. Thermostatic control kept the temperature of the subsolution constant within $\pm 0.5°$. The sensitivity of the apparatus was 0·1 dyne/cm.

Turbidity measurements were made in a simple turbidimeter (Holt & Chalk, 1953) consisting of a source of white light mounted to send a beam through one of two glass cells, either of which could be slid into the light path. That part of the light which is scattered at about 90° to the incident beam falls on a potoelectric cell set above the glass cells; the output of the photoelectric cell is measured by a sensitive galvanometer.

Heptadecylamine. Heptadecylamine was first spread as the free base, but, because its solutions rapidly combined with CO_2, the hydrochloride was preferred. Films spread from the free base gave force/area curves identical with those given by the hydrochloride. The amine or its hydrochloride was spread from 0·07 % (w/v) solution in benzene–methanol (5:2, v/v) on to subsolutions (0·01 M-NaCl, 0·005 M-$NaHCO_3$; pH adjusted with HCl or NaOH). After the films had been studied over a pH range from 3 to 12 on these substrates, the amine was spread on solutions containing, in addition to the 0·01 M-NaCl and 0·005 M-$NaHCO_3$, 0·005 M silicic acid. To compare the effects of different buffers, a 0·005 M sodium phthalate buffer was also used at pH 6·2. After the heptadecylamine had been applied to the surface, films were retained at an area of 74 sq.Å/molecule for 5 min. before measuring the force/area characteristics.

The force/area curves of heptadecylamine on silicic acid-free subsolutions are shown in Fig. 1. On alkaline solutions the films were condensed to areas, under zero compression, of between 27 and 29 sq.Å/molecule, while from pH 6 to 8 they were even more closely packed, giving a value of 24·5 sq.Å/molecule at pH 7·1. Below pH 6 they were vapour-expanded. At all pH values the films were stable up to approximately 37 dynes/cm., at which pressure they were compressed to about 20 sq.Å/molecule. With phthalate buffer at pH 6·2 the film could not be compressed below 35 sq.Å/molecule before collapsing at 35 dynes/cm.

The force/area curves of the amine on silicic acid subsolutions are shown in Fig. 2. Above pH 10 they appear to be identical with those of the amine on silicic acid-free subsolutions. At pH 9 the films were expanded and a surface pressure of just over 50 dynes/cm. was required to compress them to 20 sq.Å/molecule. From pH 9 to 5 the films were partly solidified at large areas/molecule and on compression they collapsed at about 15–20 dynes/cm. leaving shearing films. Below pH 5 no inter-

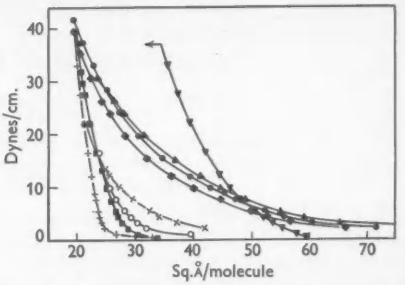

Fig. 1. Force/area curves of heptadecylamine (0·01 M-NaCl, 0·005 M-$NaHCO_3$), 20°. pH: ■, 9·0; +, 7·2; ○, 5·5; ×, 5·1; ◆, 4·4; ●, 4·0; ▲, 3·0; ▼, 6·2 on 0·005 M phthalate buffer.

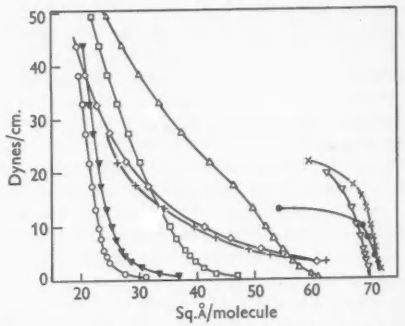

Fig. 2. Force/area curves of heptadecylamine (0·01 M-NaCl, 0·005 M-$NaHCO_3$, 0·005 M silicic acid), 20°. pH: ○, 11·0; ▼, 9·9; □, 9·0; △, 8·1; ▽, 6·6; ×, 5·7; ●, 5·0; +, 4·2; ◇, 3·4.

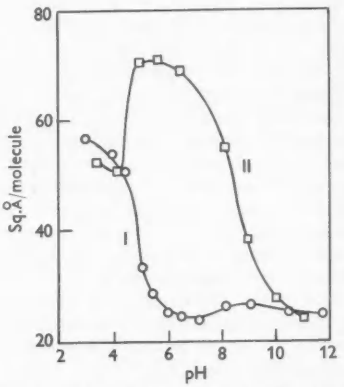

Fig. 3. The areas of heptadecylamine monolayers measured at 5 dynes/cm. plotted against the pH of the subsolution: I, subsolution (0·01 M-NaCl, 0·005 M-$NaHCO_3$); II, subsolution (0·01 M-NaCl, 0·005 M-$NaHCO_3$, 0·005 M silicic acid).

action was apparent and the vapour-expanded films deviated very little from those on silicic acid-free solutions.

Although the subsolution contained excess of silicic acid, there was a variation in the area/molecule in the solidified film when the quantity of amine spread was varied. It follows that the amount of silicic acid adsorbed depends on the initial state of expansion of the film, as would be expected if polymers are adsorbed from a polydisperse system. (Compounds have been prepared by other methods (Merrill & Spencer, 1951) in which guanidine and quaternary bases are combined with silicic acid in stoicheiometric proportions.)

For this reason all films were held at a fixed area of 74 sq.Å/molecule for 5 min. before compression. Therefore, the measured values of the area/molecule in the compressed film relate only to the particular experimental conditions used.

Fig. 3 shows the variation of molecular area of the amine with pH, measured at a surface pressure of 5 dyne/cm. and compares the areas on silicic acid with those on silicic acid-free solutions.

Myristic and stearic acids. The acids were spread from solutions in light petroleum (b.p. 60–80°) on to subsolutions of 0·05 M-NaCl or (0·05 M-NaCl, 0·025 M prepolymerized silicic acid). Myristic acid was used for pH values up to 4 (above this value the films are too soluble to form stable monolayers) and stearic acid at pH 6·7.

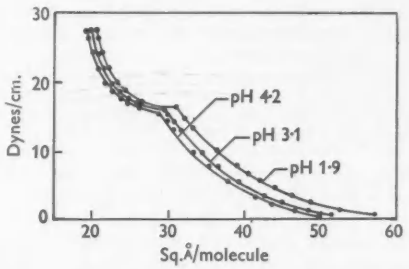

Fig. 4. Force/area curves of myristic acid on 0·05 M-NaCl.

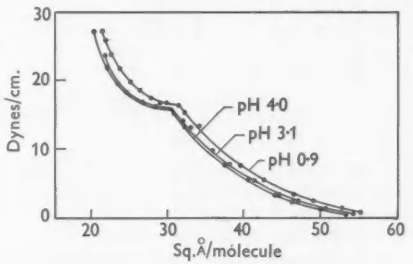

Fig. 5. Force/area curves of myristic acid (0·05 M-NaCl, 0·025 M silicic acid), 20°.

The force/area curves for myristic acid were unaltered by silicic acid (Figs. 4, 5), and there were no signs of solidification even after 12 hr. On silicic acid-free subsolutions at pH 6·7, stearic acid forms condensed films, the force/area curves of which are divisible into two linear regions, above and below 20 dynes/cm. Although silicic acid partially eliminated the lower slope so that complete compression occurred at low pressures, there was no tendency for the film to become solidified (Fig. 6).

Octadecan-1-ol. The spreading solvent used was light petroleum. Subsolutions were 0·2 M-NaCl and (0·2 M-NaCl, 0·1 M silicic acid), both at pH 6. In this case the silicic acid was not prepolymerized. The films were compressed to 45 sq.Å/molecule for 40 min. before the force/area characteristics were determined.

Curve I of Fig. 7 shows the state of the alcohol film on the sodium chloride alone (after 40 min.), and curve II on the sodium chloride–silicic acid substrate after the same time. After 40 min. it was found that the film on silicic acid was in a solid gel stage, stable to more than 50 dynes/cm. surface pressure and reversibly compressible, but unable to expand to an area greater than the original 45 sq.Å/molecule. This result, although repeated, was under suspicion since it was possible that by using such a high silicic acid concentration, gelling may have occurred at the surface of the subsolution and the effect on the alcohol film could have been simply mechanical. Therefore the experiment was repeated, making certain that the time between preparing the silicic acid and spreading the alcohol

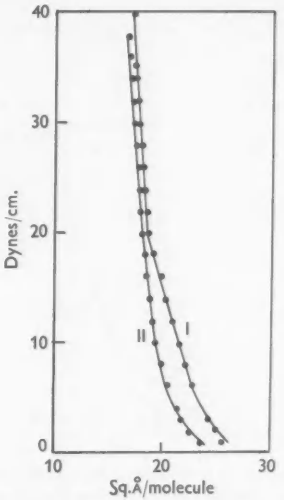

Fig. 6. Force/area curves of stearic acid at pH 6·7, 25°. I, subsolution (0·02 M-NaCl); II, subsolution (0·02 M-NaCl, 0·01 M silicic acid).

film was as short as possible, i.e. less than about 15 min., so that gelling was unlikely to occur during the experiment. Treated in this way, the films were not solid even after 1 hr., but on the other hand they were stable up to 50 dynes/cm., whereas 40 dynes/cm. is the normal collapse pressure.

Methaemoglobin. Methaemoglobin, dissolved in 0·01 M-NaCl in 1% (v/v) aqueous ethanol was spread on to (0·05 M sodium chloride, 0·005 M sodium bicarbonate). Spreading was complete in 15 min. Surface pressure/area curves were determined at a number of pH values. The variation of area with pH at the constant surface pressure of 5·5 dynes/cm. is shown in curve I (Fig. 8). Two minima occur, one at pH 4·3 and the other at pH 11·0. Between these points is a region in which the film characteristics show little variation.

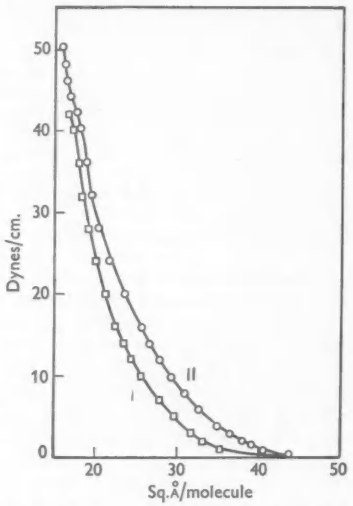

Fig. 7. Force/area curves of octadecan-1-ol, 20°. I, subsolution (0·2 M-NaCl); II, subsolution (0·2 M-NaCl, 0·1 M silicic acid).

Methaemoglobin was spread on similar subsolutions containing also 0·025 M silicic acid and the force/area characteristics were again measured after 15 min. Between about pH 4·5 and 9 spreading was incomplete and the apparent area/mg. was lower than for methaemoglobin on silicic acid-free subsolutions. The films, however, were stable at surface pressures above the normal value of about 22 dynes/cm. These apparent areas/mg. (computed from the mass of protein spread) are compared with the normal in Fig. 8.

Turbidimetric study of the influence of pH *on the interaction of silicic acid and proteins*

The protein or polypeptide (10 ml. of 0·1% (w/v) aqueous solution) was mixed with an equal volume of 0·025 M prepolymerized silicic acid. The pH was adjusted by the addition of NaOH or HCl and the volume made up to 25 ml. The light-scattering power of each suspension was then compared in the turbidimeter with that of the original silicic acid sol in comparable dilution.

As methaemoglobin is itself precipitated in acid solution, turbidity measurements were also made on a series of methaemoglobin solutions.

The turbidimeter readings of the several protein-silicic acid complexes are plotted against pH in Fig. 9, the readings being expressed as a fraction of the maximum value obtained for each protein.

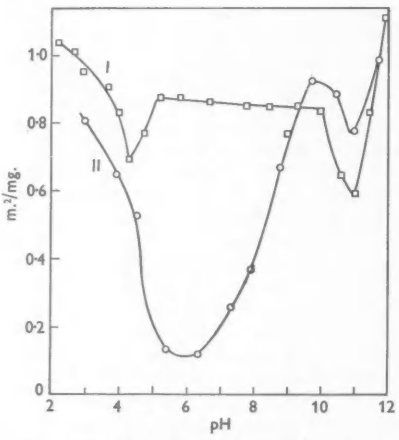

Fig. 8. The areas of methaemoglobin monolayers measured at 5·5 dynes/cm. plotted against the pH of the subsolution, 20°. I, subsolution (0·05 M-NaCl, 0·005 M-$NaHCO_3$); II, subsolution (0·05 M-NaCl, 0·005 M-$NaHCO_3$, 0·025 M silicic acid).

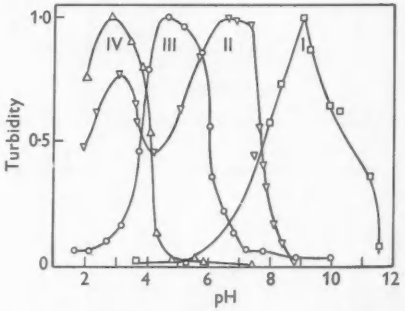

Fig. 9. The turbidities at various pH values of solutions containing protein or polypeptide to which silicic acid had been added. For details, see text.

I Poly-L-lysine
II Methaemoglobin } With silicic acid
III Serum albumin
IV Methaemoglobin alone

Bovine serum albumin. The turbidity was greatest at pH 4·6 and was immediately dispersed by an increase or decrease of 2 units of pH.

Methaemoglobin. The maximum turbidity occurred at pH 6·3–6·8. Another peak appearing at pH 3·1 coincided with that of methaemoglobin in the absence of silicic acid and is not to be regarded as due to co-precipitation of haemoglobin with silicic acid.

Poly-L-lysine. No precipitate was formed below pH 6. Unlike the rounded maxima given by the proteins, polylysine gave a sharp peak at pH 9.

DISCUSSION

Of the simple compounds studied as surface films, only heptadecylamine shows a definite interaction with polysilicic acid. The dependence of area/molecule of amine on the size of the buffering anion, demonstrated when the phthalate buffer was substituted for a carbonate buffer, indicates that the amine is associated with the anion at pH 6, and from curve I, of Fig. 3, it is evident that the amine salt is associated in the pH range 5–9. It is over this range that silicic acid is adsorbed, suggesting that the adsorption complex is an insoluble amine silicate. The concentration of silicate ions at this pH would be low (pK_a of silicic acid = 9·5, Treadwell, 1935), but their removal from the system to form the insoluble silicate layer would shift the equilibrium and induce further ionization of the acid. The formation of an insoluble silicate must then represent the main type of interaction between proteins and silicic acid. No interaction is apparent between silicic acid and the carboxyl groups.

From the turbidimetric measurements it can be seen that the interaction of silicic acid with methaemoglobin is a maximum at pH 6·5–7 and with serum albumin at pH 4·6. The surface-area measurements on the haemoglobin films indicate that a maximum interaction occurs at pH approx. 6. These values are close to the isoelectric points of the two proteins (haemoglobin, 6·8; serum albumin, 4·9), and they suggest that silicic acid is bound most strongly when the net charge on the protein is zero. On the alkaline side of its isoelectric point, the overall negative charge borne by the protein will act as a potential barrier to the approaching anions and will therefore effectively prevent any interaction. This is demonstrated by the effect of polysilicic acid on polylysine. The synthetic polypeptide bears no acidic groups other than a few α-carboxyl radicles, and it is evident that the ε-amino groups are able to react at pH values near 9, whereas in the protein the ionized carboxyl groups prevent interaction at this pH. Thus in the protein most of the lysine and arginine cannot react with silicic acid because of the repellent effect of the ionized carboxylic, phenolic and thioalcoholic groups. On the acid side of the isoelectric point the ion pairs are dissociated.

Judging by the pK_a values of the various amino acids represented in the side chains of the proteins, it seems probable that silicic acid is bound to the protein mainly by the histidine and α-amino groups.

The results of the measurements on alcohol films are not conclusive. They do not establish that silicic acid is adsorbed at an appreciable rate but they do suggest some interaction, in that the silicic acid appears to gel initially along the surface of oriented hydroxyl groups. Therefore, although silicic acid must be held mainly by the amino groups of the protein, secondary forces such as weak interactions with hydroxyl groups may also be possible. The cationic groups of the protein will attract silicate ions to the surface. The hydroxyl group cannot attract the silicic acid or silicate ions; they must reach the protein layer by diffusion and, consequently, any interaction will be a much slower process. It would seem then that the reactants are brought together primarily by the forces of electrovalency but are later anchored by subsidiary links, such as hydrogen bonds and the van der Waals forces. The analogy between silicic acid and the polysaccharidic acids is evident.

SUMMARY

1. The behaviour of methaemoglobin, heptadecylamine, myristic acid, stearic acid and octadecan-1-ol monolayers spread on buffers with and without silicic acid have been compared. The interactions with silicic acid of bovine serum albumin, methaemoglobin and poly-L-lysine were studied turbidimetrically.

2. The proteins and polylysine bind silicic acid most strongly at their respective isoelectric points.

3. Heptadecylamine reacts with silicic acid between pH's 5 and 9. Octadecanol perhaps reacts weakly. The carboxylic acids do not react.

4. It is concluded that proteins combine with silicic acid mainly through their amino groups but subsidiary links, such as hydrogen bonds and the van der Waals forces, may also be involved.

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Porphobilinogen

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Porphobilinogen was isolated by Westall (1952) from the urine of a patient with acute porphyria. A structure (I), to be discussed later, was assigned to it by Cookson & Rimington (1953) and Cookson (1953*a*) on the basis of the chemical reactions briefly recorded in these preliminary notes, and was further supported by the X-ray crystallographic investigations of Kennard (1953). In the present paper our chemical studies are described in detail, together with an improved method of isolation of porphobilinogen, and a discussion of its conversion into porphyrin.

EXPERIMENTAL

Melting points are uncorrected. Paper chromatograms of porphobilinogen and its derivatives were run using the upper layer of a mixture of *n*-butanol:water:acetic acid (4:1:4, v/v) at 17°, and developed with 1% (w/v) *p*-dimethylaminobenzaldehyde in 2·5N-HCl. Ultraviolet absorption spectra between ~210 and 400 mμ. were measured in ethanolic solution, except where another solvent is specified, with a Unicam SP500 Spectrophotometer. Maximum and minimum are printed in normal type, shoulders in italics. Infrared absorption spectra were measured on a Grubb Parsons F3A spectrometer. Samples were examined as Nujol mulls. The intensity of absorption is indicated as follows: (s), strong; (m), medium; (w), weak; ?, doubtful.

Isolation of porphobilinogen

The material used initially was kindly provided by Mr R. G. Westall, and we later isolated further quantities by his method (Westall, 1952). Then, following Brockman & Gray (1953), we substituted Deacidite FF for Dowex 2. Both methods are rather slow, and the instability of porphobilinogen under these conditions often results in low yields. Neither method is suitable for urines relatively poor in porphobilinogen. The following procedure avoids the use of exchange resin and alumina chromatography, is rapid, and gives very much better yields.

Urine containing porphobilinogen was brought to pH 4–4·5 with acetic acid. Mercuric acetate (15%, w/v) solution was added until all porphobilinogen was precipitated (negative Ehrlich reaction of the supernatant); the precipitate was collected on a Büchner funnel and washed with mercuric acetate (1%, w/v). It was then suspended in water and decomposed by H_2S. After filtration and aeration, excess of lead acetate (10%, w/v) was added. The deeply pigmented precipitate was removed and washed repeatedly with 1% (w/v) lead acetate solution. $AgNO_3$ (20%, w/v) solution was added to the combined filtrate and washings until precipitation was complete. After filtration and washing of the precipitate with 1% (w/v) $AgNO_3$, the combined filtrate and washings were treated, without prior removal of Pb and Ag ions, with a slight excess of 15% (w/v) mercuric acetate solution. This mercury precipitate was almost white; it was collected by centrifuging and decomposed by passing H_2S without the addition of further fluid. HgS was easily removed by centrifuging from the pale yellow supernatant, which was freed from H_2S by aeration and then adjusted to pH 4 with aqueous NH_3. Porphobilinogen crystallized immediately in almost colourless, well formed, crystals. After chilling, the solution was filtered and the porphobilinogen washed first with water containing a little acetic acid, and then with acetone. Further quantities could usually be obtained by the same method by extracting the final HgS precipitate with a little water. Yields were about 60%.

Porphobilinogen was recrystallized for analysis by solution in 0·5N-NH_3 and addition of N acetic acid to pH 4. The crystals of *porphobilinogen monohydrate* were washed free from salt and were then air-dried. (Found: C, 49·3; H, 7·0; N, 11·4. $C_{10}H_{14}O_4N_2$, H_2O requires C, 49·2; H, 6·6; N, 11·5%.) *Porphobilinogen* (*anhydrous*) was obtained by drying over P_2O_5 at 20°/0·1 mm. for 3 days. (Found: C, 52·8; H, 6·8; N, 12·1. $C_{10}H_{14}O_4N_2$ requires C, 53·1; H, 6·2; N, 12·4%.) When exposed to the atmosphere it ceased to increase in weight after 1 hr. (increase in weight, 7·9%. Theory for 1 molecule of water, 7·3%). The analysis of porphobilinogen and its salts presented considerable difficulty on account of both their instability and hydration. Porphobilinogen was optically inactive at all pH values examined.

Estimation of porphobilinogen

Vahlquist (1939) described the determination of porphobilinogen in urine by means of the Ehrlich aldehyde reagent, but the definition of his porphobilinogen unit is ambiguous. We interpret it as follows: a porphobilinogen solution, which when mixed with an equal volume of Ehrlich reagent (2%, w/v, in 5N-HCl) has $E^{1\,\mathrm{cm.}}_{552\,\mathrm{m}\mu} = 0{\cdot}545$, contains 1 unit/ml. This is measured against a blank cell containing equal volumes of the solution to be determined and 5N-HCl. Under these conditions maxima are at 552 and 530 mμ. Crystalline porphobilinogen (as normally obtained) assays 130 units/mg. Here and subsequently weights refer to the monohydrate.

Derivatives of porphobilinogen

Porphobilinogen hydrochloride. Westall (1952) observed that porphobilinogen forms a hydrochloride soluble in water but sparingly so in 2N-HCl. This salt is convenient for

the further purification of the crude base, particularly that obtained by the chromatographic methods, which is often deeply coloured and contains some uroporphyrin. The crude material was dissolved in 2N-HCl (1 ml./50 mg.) by warming to 45–50°. Filtration, if necessary, was carried out rapidly, and the solution immediately cooled (ice-NaCl). The hydrochloride, which crystallized in almost colourless needles, was filtered from the dark mother liquors, washed sparingly with ice-cold 2N-HCl and then with dry acetone. Yield about 70%. Recrystallization of the hydrochloride from 2N-HCl was conveniently carried out in a suitably jacketed no. 3 sintered glass funnel. (Found: C, 42·9; H, 6·3; N, 9·6; Cl, 12·8. $C_{10}H_{14}O_4N_2$, HCl, H_2O requires C, 42·8; H, 6·1; N, 10·0; Cl, 12·7%. Gain in weight on exposure to the atmosphere of a sample which had been kept over P_2O_5 at 20°/0·1 mm. for 3 days, 6·3%. Theory for one molecule of water, 6·4%.)

Porphobilinogen hydrobromide. This was prepared in the same way as the hydrochloride and is rather more soluble. Both the hydrate and anhydrous salt have been observed to crystallize together from the same solution (Kennard, 1953). The analytical sample was dried for 3 days over P_2O_5 at 20°/0·1 mm. (Found: C, 40·3; H, 5·6; N, 9·1; Br, 26·5. $C_{10}H_{14}O_4N_2$, HBr requires C, 39·1; H, 4·9; N, 9·1; Br, 26·0%. Loss in weight of hydrate on drying, 5·3%; gain in weight on exposure of dried sample to atmosphere, 5·6%. Theory for one molecule of water, 5·5%.)

Acyl derivatives of porphobilinogen. Porphobilinogen (34·9 mg.) was dissolved in N-NaOH (1 ml.) and acetic anhydride (0·1 ml.) added. Efforts to isolate a solid derivative after careful acidification were not successful, although the presence of material believed to be *N*-acetylporphobilinogen was recognized by paper chromatography of the much diluted reaction mixture. Ehrlich's reagent revealed only a single spot with R_F 0·79. The same result was obtained when triethylamine was used in place of NaOH. *N*-Benzoylporphobilinogen was similarly tentatively identified as the only Ehrlich-positive product in a small-scale experiment in which benzoyl chloride was used instead of acetic anhydride (R_F, 0·92).

Porphobilinogen lactam (II). Acetic anhydride (0·2 ml.) was added in portions to a cold solution of porphobilinogen (51 mg.) in water (1·9 ml.) and pyridine (0·6 ml.). The product began to separate after 15 min.; it was filtered off and washed several hours after. It was practically insoluble in the usual solvents, but could be dissolved in N-NH_3 and reprecipitated by 0·5N acetic acid. The *lactam* (25·7 mg., 59%) formed colourless leaflets which began to darken above 200° and melted with effervescence at *c.* 285° (R_F, 0·65). (Found: C, 57·6; H, 5·9; N, 12·9. $C_{10}H_{12}O_3N_2$ requires C, 57·7; H, 5·8; N, 13·5%.) Ultraviolet absorption in 0·01N-NaOH: $\lambda_{max.}$ at *c.* 210 mμ. (ϵ, 7500). Infrared absorption: NH stretching *3290*, 3200 (s), 3100 (w) cm.$^{-1}$; OH stretching ~2250 cm.$^{-1}$; CO stretching 1698 (—COOH), 1637 (—CONH—) cm.$^{-1}$; second amide band 1612 cm.$^{-1}$. Both the solid and its solutions fluoresced a brilliant blue under a Wood's lamp. The Ehrlich colour given by the lactam appeared immediately, in contrast to that from porphobilinogen which only develops slowly. Paper chromatography of the mother liquor from the reaction showed the presence of more of the lactam and also of *N*-acetylporphobilinogen.

The lactam, identical in all respects with the above, was also formed when benzoyl chloride was used instead of acetic anhydride, but the yield of lactam was lower (29%). Toluene-*p*-sulphonyl chloride was even less satisfactory.

The lactam was unchanged by solution in N-NaOH and further treatment with acetic anhydride.

Methyl ester of porphobilinogen lactam. This was prepared by treatment of the finely powdered lactam (40 mg.) with excess of diazomethane (made from 3 g. nitrosomethylurea) in ether (40 ml.) containing methanol (2 ml.). After 2 days at 4° the solvent was removed *in vacuo* and the residue recrystallized from ethanol, sublimed at 230°/0·1 mm., and the sublimate (25·9 mg.) again recrystallized from ethanol (7 ml.). The *ester* formed colourless platelets (24·5 mg.) which showed the same blue fluorescence as the free acid, and which melted at 248–250° (R_F, 0·83). (Found: C, 59·6; H, 6·6; N, 12·6. $C_{11}H_{14}O_3N_2$ requires C, 59·4; H, 6·3; N, 12·6%.) Ultraviolet absorption: $\lambda_{max.}$ at *c.* 210 mμ. (ϵ, 7500).

2-Acetyl porphobilinogen lactam methyl ester (III). A solution of the lactam ester (8·2 mg.) in boiling acetonitrile (4 ml.) was rapidly cooled; a stream of dry HCl was immediately passed into the supersaturated solution for 8 min., and the mixture was set aside overnight. The solid (0·35 g.) which remained after removal of excess of nitrile under reduced pressure, consisting largely of a salt derived from acetonitrile itself, was dissolved in water (2 ml.) and a sample suitably diluted for measurement of its spectrum. Although the product from the reaction between HCl and acetonitrile alone did not absorb light in the range studied, an aqueous solution of equivalent concentration was used in the blank cell. The acidic reaction solution had an absorption maximum at 331 mμ., which was assumed to be due to the ketimine hydrochloride, for the spectrum changed when the solution was made alkaline: The spectrum rapidly changed further as hydrolysis of the ketimine to the acetyl compound took place. Hydrolysis was complete after 3 hr. at 17° when the absorption maximum was at 306 mμ. If ϵ is 20000 at this wavelength (Cookson, 1953*b*) and the reaction had gone in quantitative yield, the optical density indicated a molecular weight of about 300 for the lactam ester (theory, 222). This upper limit to the molecular weight provided further support for the formulation of porphobilinogen as a monopyrrole.

2:4-Dinitrophenylhydrazine (13·2 mg.) was added to the main bulk of the reaction mixture (which was Ehrlich-negative). After heating the solution for a short time, blood-red needles of the 2:4-*dinitrophenylhydrazone* of the acetyl compound separated (2·7 mg.). The m.p. was indefinite, with decomposition above 215°. (Found: N, 19·2; $C_{19}H_{20}O_7N_6$ requires N, 18·9%.)

Ketolactam (IV). The free lactam (II) (37·2 mg.) was stirred with polyphosphoric acid (2·0 g.) at 95° for 5 min. The lactam dissolved and the mixture became red. Stirring was continued, while the temperature was raised to 110° during the next 10 min. and the colour of the mixture faded to yellow. After cooling the mixture, water was added and the precipitate (29 mg. 85%) washed with dilute aqueous NH_3 and with water. The *ketolactam*, which was insoluble in the usual organic solvents, was recrystallized from water (25 ml.). It formed colourless prisms (22·3 mg.) which began to sublime at 310° and then at *c.* 330° to decompose without melting (Found: C, 63·4; H, 5·4; N, 14·7. $C_{10}H_{10}O_2N_2$ requires C, 63·2; H, 5·3; N, 14·7%.) Ultraviolet and infrared absorption values are given in Table 1. ($\lambda_{max.}$ in aqueous solution, 288·5 mμ.)

Attempts to cyclize (II) to (IV) by means of conc. H_2SO_4, $SOCl_2$, $POCl_3$ or anhydrous HF were unsuccessful.

Table 1. *Comparison of ultraviolet and infrared absorption characteristics in ethanol of the ketolactam (IV) with those of model compounds*

	Ketolactam (IV)	1-Oxo-2:3-dihydropentindole (VI)	1-Oxo-1:2:3:4-tetrahydrocarbazole (V)
M.p.	>330°	250°	169°
	Ultraviolet absorption		
$\lambda_{max.}$ (mμ.)	*255* (ε, *8 300*) 284 (ε, 20 600)	234 (ε, 19 400) 300 (ε, 25 500)	237 (ε, 16 200) 308·5 (ε, 23 000)
$\lambda_{min.}$ (mμ.)	223 (ε, 1 200)	214 (ε, 7 750) 258 (ε, 1 300)	220 (ε, 8 800) 264 (ε, 1 000)
	Infrared absorption		
In NH stretching region ν (cm.$^{-1}$)	*3200*? 3145 (s) Broad 3060 (s) 2780 (m) 2700 (w) 2620 (w)	3205 (s) — 3060 (m) 2780 (w) — —	3300 (s) *3200* (w) — — — —
In CO region ν (cm.$^{-1}$)	1654 (s) Very broad	1656 (s) 1620 (m)	1645 (s) *1620*?

Synthetic experiments

1-Oxo-1:2:3:4-tetrahydrocarbazole (V) was prepared from γ-3-indolylbutyric acid by cyclization with polyphosphoric acid for 10 min. at 80°. A sample of 1-oxo-2:3-dihydropentindole (VI) was kindly provided by Dr J. Elks (see Elks, Elliott & Hems, 1944). Ethyl 2:3-dimethylpyrrole-5-carboxylate was prepared by the method of Fischer & Fink (1948). Its hydrolysis and decarboxylation provided 2:3-dimethylpyrrole.

5-β-Chloropropionyl-2:3-dimethylpyrrole. 2:3-Dimethylpyrrole (2·93 g.) dissolved in ether (10 ml.) was added to a Grignard reagent prepared from bromobenzene (4·33 g.) and Mg (0·74 g.) in ether (20 ml.). The solution was refluxed for 30 min. before being cooled to 0°, while a solution of β-chloropropionyl chloride (3·54 g.) in ether (15 ml.) was added. A deeply coloured viscous oil separated towards the end of the addition. After being refluxed for 1 hr., the mixture was again cooled to 0° before hydrolysis with NH_4Cl solution. The *chloroketone*, which was only slightly soluble in ether, was filtered off and recrystallized from ethanol. It formed white leaflets which melted to a carmine oil at 119°. (Found: C, 58·2; H, 6·6; N, 7·5. $C_9H_{12}ONCl$ requires C, 58·2; H, 6·5; N, 7·6%.) Ultraviolet absorption: $\lambda_{max.}$ 256 (ε, 4200) and 313 mμ. (ε, 18 800); $\lambda_{min.}$ 225 (ε, 1100) and 273 mμ. (ε, 3100). Attempts to cyclize the chloroketone with polyphosphoric acid, $SnCl_4$, or $AlCl_3$ in tetrachloroethylene were unsuccessful.

2:3-Dimethyl-5-pyrryl vinylketone. The chloropropionylpyrrole (745 mg.) was boiled for 20 min. with excess of pyridine after which the latter was removed under reduced pressure. The deliquescent quaternary salt was dissolved in a small volume of water, to which dilute NaOH was added dropwise. When no more of the *vinyl ketone* was precipitated, it was filtered off and washed with water. It separated from light petroleum (b.p. 60–80°) in pale lemon rhombs (396 mg.) m.p. 142–143°. (Found: C, 72·5; H, 7·6; N, 9·5. $C_9H_{11}ON$ requires C, 72·5; H, 7·4; N, 9·4%.) Ultraviolet absorption: $\lambda_{max.}$ 210 (ε, 4600), 237·5 (ε, 5000), 346 (ε, 16 600), and *275* mμ. (ε, *2700*); $\lambda_{min.}$ at 222 (ε, 4100), and 262 mμ. (ε, 2000). Attempts to cyclize the vinyl ketone were not successful.

Ethyl 2:3-dimethyl-4-morpholinomethylpyrrole-5-carboxylate. A mixture of ethyl 2:3-dimethylpyrrole-5-carboxylate (10 g.), morpholine (6·24 g.), conc. HCl (6·3 ml.), 40% (w/v) aqueous formaldehyde (6·0 ml.) and ethanol (30 ml.) was boiled under reflux. After 5 hr. the ethanol was removed under reduced pressure and water was added. The unchanged pyrrole which was precipitated, was filtered off, and boiled again with the same reagents. On making the filtrate alkaline with NaOH the *morpholino compound* precipitated as an oil which rapidly solidified. It recrystallized from ethanol in colourless needles, m.p. 137–138° (2·5 g.; the recovered material provided another 2·47 g. together with 5·4 g. still unchanged pyrrole). (Found: C, 63·2; H, 8·3; N, 10·4. $C_{14}H_{22}O_3N_2$ requires C, 63·2; H, 8·3; N, 10·5%.) Ultraviolet absorption: $\lambda_{max.}$ 243 (ε, 4500) and 287 mμ. (ε, 17 000); $\lambda_{min.}$ 220 (ε, 2300) and 255 mμ. (ε, 3900). In acid ethanol: $\lambda_{max.}$ 238 (ε, 3400) and 290 mμ. (ε, 16 500); $\lambda_{min.}$ at 215 (ε, 1600) and 263 mμ. (ε, 2900). The use of paraformaldehyde in place of formaldehyde solution in the Mannich reaction was less satisfactory.

The *methiodide* of the morpholino compound separated from a mixture of ethyl acetate and ethanol in slightly yellow crystals which melted at 180° with decomposition and previous darkening. (Found: C, 44·3; H, 6·3; N, 6·8. $C_{15}H_{25}O_3N_2I$ requires C, 44·1; H, 6·1; N, 6·9%.)

Ethyl 4-2′-tris(ethoxycarbonyl)ethyl-2:3-dimethylpyrrole-5-carboxylate. An ethanolic solution (5 ml.) of the methiodide prepared from the above morpholino compound (900 mg.) was added to ethanol (10 ml.) in which Na (78 mg.) and tris(ethoxycarbonyl)methane (1·57 g.) had been dissolved. The mixture was refluxed for 6 hr. On the next day the ethanol was distilled off under reduced pressure, and water added to the residue, which was then made just acid, before extraction with ether. Evaporation of the washed and dried (Na_2SO_4) extract left a viscous oil. A small sample which was obtained crystalline by distillation at a high temperature at 0·1 mm. was used to seed the main bulk. The *product* (709 mg.) was washed free from traces of oil with light petroleum (b.p. 40–60°) and recrystallized from light petroleum (b.p. 60–80°) containing a trace of benzene. The highly refracting rhombs melted at 83–84° (570 mg.). (Found: C, 58·5; H, 7·0; N, 3·5. $C_{20}H_{29}O_8N$ requires C, 58·4;

H, 7·1; N, 3·4%.) Ultraviolet absorption: $\lambda_{max.}$ at 245 (ϵ, 4600) and 288 mμ. (ϵ, 17000); $\lambda_{min.}$ at 222 (ϵ, 3100) and 256 mμ. (ϵ, 4200).

Ethyl 4-2'-bis(ethoxycarbonyl)ethyl-2:3-dimethylpyrrole-5-carboxylate. A solution of the methiodide derived from the above morpholino compound (5·3 g.) in ethanol (20 ml.) was added to one of ethyl malonate (6·4 g.) in ethanol (30 ml.) in which Na (0·46 g.) had been dissolved. The mixture was refluxed for 6 hr. and then worked up as above. The excess of ethyl malonate was removed at 120°/0·2 mm., but the residue did not crystallize and chromatography on Al_2O_3 in ether–light petroleum (b.p. 40–60°) (20:80, v/v) was necessary. The *pyrrole* thus purified separated from light petroleum (b.p. 60–80°) in colourless crystals (3·5 g., 52%) which melted at 63–64°. (Found: C, 59·8; H, 7·7; N, 3·8. $C_{17}H_{25}O_6N$ requires C, 60·2; H, 7·4; N, 4·1%.) Ultraviolet absorption: $\lambda_{max.}$ at 247 (ϵ, 4500) and 287 mμ. (ϵ, 17 700); $\lambda_{min.}$ at 220 (ϵ, 2400) and 255 mμ. (ϵ, 4200).

Neither the hydrolysis of this compound nor that of the foregoing tris(ethoxycarbonyl)ethyl derivative was investigated in detail. Both gave free acids which were extremely soluble in water and could not be isolated. Hydrolysis of the malonic ester derivative (1·28 g.) by boiling water (4 ml.) containing ethanol (1 ml.) and NaOH (0·73 g.) for 4 hr., followed by the addition of HCl to pH 3·5, gave a solution of the polycarboxylic acid, which was decarboxylated to haemopyrrole carboxylic acid (0·10 g.) by heating to 160° in a Carius tube for 6 hr. Much tar was formed, and doubtless better conditions for the decarboxylation could be found.

Conversion of porphobilinogen into uroporphyrin

Conversion in acid at 100°. A solution of porphobilinogen (25 mg.) in 0·5 N-HCl (50 ml.) was heated at 100° for 20 min. The amount of porphyrin, which was estimated spectrophotometrically in a suitably diluted sample, was found to increase on exposure to air, as the initially formed porphyrinogen became oxidized. Air was drawn through the main solution to complete this oxidation; at the end of the reaction the solution contained 16·5 mg. uroporphyrin (77·5% yield) estimated spectrophotometrically. The porphyrin was precipitated at pH 3·1 (sodium acetate) and removed by centrifuging. The brown mother liquor was re-acidified and the trace of porphyrin (15·6 μg.) estimated; this solution had an absorption band at 480 mμ. and gave an intense green fluorescence with zinc acetate (cf. Brockman & Gray, 1953). A paper chromatogram of a sample of the unpurified porphyrin precipitate run in lutidine (Nicholas & Rimington, 1949, 1951) showed only an octacarboxylic porphyrin spot. Esterification of the bulk of the porphyrin with methanolic HCl gave a quantitative yield of the octamethyl ester (18·8 mg.) which melted at 258–260°. The Falk & Benson (1953) method of chromatography showed only a single spot in the uroporphyrin III position with very slight tailing.

A sample (8 mg.) of this uroporphyrin was decarboxylated in the usual way (Fischer & Zerweck, 1924); the yield was 70%, as determined photometrically. The porphyrin was isolated and esterified with methanolic HCl, and the absence of substances other than coproporphyrins was demonstrated by chromatography on Al_2O_3 (Brockmann grade V) in benzene–$CHCl_3$. Paper chromatograms run by the method of Chu, Green & Chu (1951) on samples, both before and after the Al_2O_3 chromatography, showed only single spots with very slight tailing in the coproporphyrin III position. The chromatographed coproporphyrin ester separated from methanol–$CHCl_3$ in needles with the appearance of coproporphyrin III ester; the mother liquor was only faintly coloured. The melting point of this material was taken on a micro hot stage, the sample being squeezed between coverslips so that it was translucent. Individual needles melted between 132 and 145°, but much solid remained up to ca. 160° and a few crystals remained until 190°. When the temperature was lowered the oil readily recrystallized in long pleochroic laths, which began to melt again at *c.* 160°, but did not melt completely until 197°. This behaviour was visible both in ordinary light and between crossed Nicols. A sample of coproporphyrin III tetramethyl ester prepared from *Corynebacterium diphtheriae* melted at 148–150° and resolidified while the temperature was raised; it melted again at 170–177°.

Conversion at various pH *values at* 20°. Four solutions, made up with the compositions shown in Table 2, were set aside in the dark for 17 days. The time after which a definite pink fluorescence became visible when illuminated with a Wood's lamp gave a rough indication of the rate of porphyrin formation. At the end of 17 days the unchanged porphobilinogen still present was estimated as described above and the uroporphyrin was estimated by the method of Rimington & Sveinsson (1950). The bulk of each solution

Table 2. *Conversion of porphobilinogen into uroporphyrins in various solutions at* 20°

Pbg = porphobilinogen

Composition	pH	Time of appearance of fluorescence (days)	Uroporphyrin yield in (μg./mg. Pbg)	Unchanged Pbg (mg.)	Apparent composition of uroporphyrin mixture (%)
A. 2·4 mg. Pbg, 50 mg. Na_2CO_3, 0·5 ml. water, 4·5 ml. normal urine	>10	7	65	0	'I' 60 'III' 40
B. 2·3 mg. Pbg, approx. 8·1 ml. HOAc, 4·75 ml. normal urine, approx. 1·2 ml. 2 N-NaOH	6·5	2	160	0	'I' 0 'III' 100
C. 2·0 mg. Pbg, 5·0 ml. 0·2 N-NH_3	>10	4	80	0	'I' 50 'III' 50
D. 2·2 mg. Pbg, 5·0 ml. 0·5 N-HCl	<1	No fluorescence purple pigment formed	0·35	1·0	Not enough material

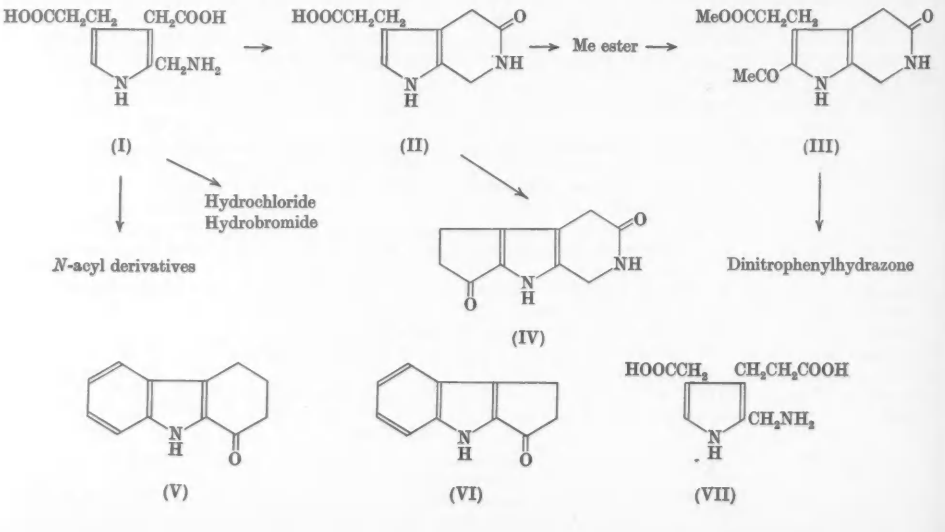
was then adjusted to pH 4·2, and the porphyrin in each adsorbed on talc. The porphyrins were esterified and purified by chromatography on MgO as described by Nicholas (1951), and then run on paper chromatograms in kerosene–dioxan (Falk & Benson, 1953). For reasons outlined below (see Discussion) the results have been expressed in terms of uroporphyrin I and III mixtures which give similar patterns (Table 2), although in the case of *B* and *C* the strongest fluorescence of the 'III' spot was on the low R_F side of the spot, whereas pure uroporphyrin III gives symmetrical fluorescence.

DISCUSSION

Structure of porphobilinogen

A close structural connexion between porphobilinogen and urophorphyrin has been evident since Waldenström & Vahlquist's (1939) demonstration of the formation of uroporphyrin when solutions of porphobilinogen are heated under suitable conditions. This transformation suggested that the substituents of any pyrrole ring present in porphobilinogen must be acetic acid and propionic acid residues, respectively, for oxidation of uroporphyrin yields carboxylated haematinic acid (MacDonald, 1952*a*). There remained many possibilities for the structure of porphobilinogen. The reactions by which we have definitely established its structure as 2-aminomethyl-4-2′-carboxyethyl-3-carboxymethylpyrrole (I) are summarized in the chart and described in the experimental part. The lactam (II) is a key compound, and its formation by treatment of porphobilinogen dissolved in aqueous pyridine with an acylating agent is of particular interest. It is evident that a mixed anhydride between the acetic acid side chain of porphobilinogen and the acylating agent is an intermediate in this reaction. For cyclization to take place this mixed anhydride must cleave in the appropriate way, and in addition the amino group must exist as the free base. The dual effects of pH and nature of the acylating agent were not studied, but the production of (II) in 59% yield under our conditions shows that the reversible attack on the carboxyl group by acetic anhydride is very much more rapid than acetylation of the amino group. This is not the case when the reaction is carried out in sodium hydroxide solution, for then the *N*-acetyl derivative is apparently the exclusive product.

There was a possibility that porphobilinogen had a structure (VII) similar to (I), but with the positions of the two β-substituents reversed; this would have led to a seven-membered lactam ring with an acetic acid side chain on the other side of the pyrrole nucleus. Such a structure, which is compatible with all the other reactions, was excluded by cyclization of (II) to the ketone (IV) by means of polyphosphoric acid. The only neutral crystalline product, which could have been derived from the alternative seven-membered ring structure for the lactam (VII), would have been an eight-membered ring diketone formed by condensation of pairs of such molecules. This unlikely formulation is inconsistent with the properties of our product, for, being unstrained, it would have been expected to have had the ultraviolet absorption of a normal 2-acyl pyrrole (e.g. 2-acetyl-4-ethyl-3:5-dimethylpyrrole, $\lambda_{max.}$ 308 mμ. Cookson, 1953*b*). In fact, our product has maximum absorption at 284 mμ., i.e. at a wavelength about 20 mμ. shorter than this. A hypsochromic shift relative to the acyclic compounds is well



known in the case of the *cyclo*pentenones (Gillam & West, 1942), and is due to the strain in a five-membered ring (French & Wiley, 1949). 1-Oxo-1:2:3:4-tetrahydrocarbazole (V) and 1-oxo-2:3-pentindole (VI), the ultraviolet absorption data for which are shown in Table 1, further illustrate the effect, but being indoles, naturally neither has absorption identical to that of the pyrrole (IV). Individual groups in the same environment have characteristic absorption in the infrared, irrespective of the structure of distant parts of the molecule, and therefore the direct comparison of the infrared spectra of the indoles with that of (IV) is permissible. The resemblance of the infrared spectrum of (IV) to the five-membered rather than to the unstrained six-membered indole derivative is significant, but this evidence is less impressive than that from the ultraviolet owing to the necessity of working with nujol mulls, and to the overlap in (IV) of the amide band at 1640 cm.$^{-1}$ with that due to the keto group. Originally it had been intended to prepare 2:3-dimethylpyrrolo-(4:5-3′:2′)-*cyclo*pentenone as a model compound. The attempts to synthesize this ketone, which are described in the experimental section, were not encouraging, and so the indoles mentioned above were used instead.

It may be noted in connexion with these experiments that although an electron-attracting group in the α-position of a pyrrole prevents substitution in the adjacent free β-position by the Gattermann-Hoesch reaction (MacDonald, 1952*b*), such a pyrrole can still take part in the Mannich reaction (cf. Treibs & Ott, 1953).

The p*K* values of porphobilinogen measured by Granick & Bogorad (1953) are those to be expected for structure (I) (cf. Branch & Calvin, 1941).

δ-Aminolaevulinic acid ($H_2N.CH_2.CO.CH_2.CH_2.CO_2H$), a specific precursor of haem (Shemin & Russell, 1953) and of porphyrins (Neuberger & Scott, 1953) was shown by Dresel & Falk (1953) to be enzymically converted into porphobilinogen. The structure I for the latter is readily intelligible when seen as the result of the condensation of two molecules of δ-aminolaevulinic acid: the alternative structure VII would not be readily produced.

Waldenström porphyrin

Urines from cases of acute porphyria suffering attacks invariably contain porphobilinogen. Not infrequently uroporphyrin is absent from the freshly voided urine but forms from porphobilinogen more or less rapidly on standing. Such uroporphyrins have all been termed 'Waldenström porphyrins', notwithstanding variations of techniques of preparation and isolation. They are in fact chemical artifacts. Failure to appreciate this point and, possibly, the influence of the concept of the dualism of the porphyrins have led to some of the present confusion. Much work has been devoted to the study of Waldenström porphyrins employing such methods of separation and identification of porphyrins and their isomers as are available. In the absence of pure specimens of all four uroporphyrin isomers it has not been possible to test these methods directly on a known complex mixture.

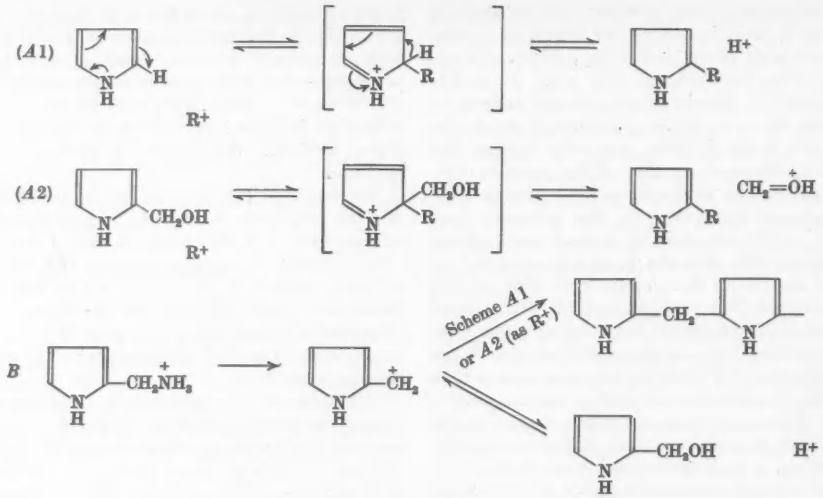
Waldenström (1935) and Mertens (1936) identified the porphyrin from acute porphyria urines as uroporphyrin III, Grinstein, Schwartz & Watson (1945) consider it to be uroporphyrin I together with a heptacarboxylic porphyrin, while Nicholas & Rimington (1953) suggested on the basis of results obtained with several techniques that it was a mixture of about 75% uroporphyrin III and 25% uroporphyrin I.

We have as yet developed no new method of porphyrin isomer analysis, but certain predictions can be made on purely chemical grounds, which are relevant to this problem. Before the isolation of porphobilinogen, the porphyrin derived from it was the only tangible clue to its structure; now the position is reversed.

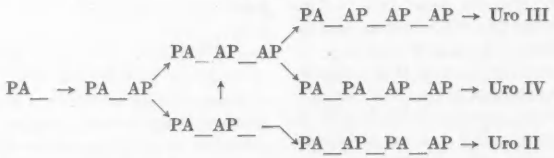
Formation in vitro *of porphyrins from pyrroles of the type of porphobilinogen*

Electrophilic attack on a pyrrole will tend to take place at the point of highest negative charge, but the reaction rate, being controlled by the energy of activation, will be favoured by a transition complex of low energy. The reaction, therefore, normally takes place by direct displacement so that the pyrrole ring system may be retained (i.e. pyrrolenines are not formed). The group replaced must be able to give up an electron pair and separate as a cation or a combination of neutral and positive fragments. Such groups include —H, —CH_2OH, —CH_2NH_2 or pyrrylmethylene, but not alkyl or —$CH_2NH_3^+$. The term 'free position' is customarily given only to one bearing a hydrogen atom, but it must be remembered that positions are not 'blocked' if substituted by other groups which are readily replaceable. Although there is no fundamental difference between condensations involving displacement of hydrogen or of a larger group, it will be convenient for the following discussion to call these two reactions of the nucleophilic component *A* 1 and *A* 2 respectively.

A compound such as porphobilinogen which contains two replaceable groups can react in several ways so that the product of each individual step, let alone of the condensation of four molecules into a porphyrin, cannot be predicted in the absence of data on reaction rates, etc. Moreover, these and the reaction mechanism, itself are doubtless dependent on pH and many other factors (e.g. the —CH_2NH_2 group can react according to *A* 2 only as free base).



Scheme *C*



It is, nevertheless, of value to discuss the composition of the isomeric porphyrin mixtures which would be obtained under various circumstances. We have experimentally observed the formation of uroporphyrinogen as an intermediate. Only the condensation in acid solution is discussed, although obviously a parallel scheme would apply for base catalysis, where the initial step would be removal of a proton from the pyrrole nitrogen. The acid-catalysed reaction is illustrated by the S_E1 reaction in which porphobilinogen first loses ammonia and the resulting carbonium ion either attacks another pyrrole acting as an electrophilic component or reacts with water (scheme *B*).

If the α-hydrogen atom is more easily displaced than aminomethyl or hydroxymethyl, reactions according to *B* and *A* 1 will lead to uroporphyrinogen I and after oxidation to uroporphyrin I.

If the aminomethyl or hydroxymethyl side chain is displaced in preference to the α-hydrogen atom (*A* 2), but the latter in preference to α-pyrrylmethylene (*A* 1) the product will be a mixture of uroporphyrins III and IV with a small amount of II (assuming equal speeds of reaction). Inspection of scheme *C* in which the porphobilinogen unit is abbreviated PA__ (P indicating the propionic and A the acetic acid residues in the ββ′ positions and the horizontal dash the link between units) will show that even if reaction *A* 2 did occur to some extent throughout the synthesis, it would have little effect on the final products, since such reactions would in most cases lead to compounds which are already intermediates in this scheme. (R in *A* 1 or *A* 2 is an α-pyrrylmethylene or the cation formed by the addition of a proton to formaldehyde).

Actual examples of a similar order of ease of substitution of groups by electrophilic reagents have been observed in phenols (which owing to the operation of similar electronic displacements are analogous to pyrroles). For example, Ziegler & Zigeuner (1949) and Ziegler (1951) investigating the reaction between diazonium salts and hydroxybenzyl alcohols found cases in which the ease of replacement of groups was in the order —CH_2OH> —H *ortho* to phenolic —OH> *ortho*hydroxybenzyl.

A proton could also act as the substituting reagent, and although reaction *A* 1 (R=H) would cause no change, reaction *A* 2 would yield formaldehyde and pyrrole-β-acetic-β′-propionic acid. The reactivities of the α-positions of this compound are probably very similar, so that the equilibrium mixture obtained by this mechanism would contain comparable amounts of the isomeric α-hydroxymethylpyrroles. If such isomerization is much more

rapid than the condensation between pyrroles, the pairs of side chains in the final porphyrin will be arranged at random, and a mixture of isomeric uroporphyrins in the proportions $\frac{1}{8}$ I, $\frac{1}{8}$ II, $\frac{1}{2}$ III, and $\frac{1}{2}$ IV will be obtained, irrespective of the details of condensation. This distribution of isomers arising from the random condensation of opso-type pyrroles has been independently noted by S. F. MacDonald (personal communication).

The discovery by Corwin & Andrews (1937) of the formation of tripyrrylmethanes during the synthesis of dipyrrylmethenes from aldehydes and pyrroles with a free α-position, which can then split into any of several different dipyrrylmethenes, is often quoted as a cause of isomerization. Redistribution of the charge on the salt of the dipyrrylmethene enables the bridge carbon atom to acquire a positive charge; and reaction according to *A* 1, where this cation is represented by R^+, sets up the equilibrium with the tripyrrylmethane. It is obvious that a dipyrrylmethane could not take part in such a reaction as the substituting reagent, and that therefore this mechanism does not concern the lower state of oxidation considered throughout the foregoing discussion.

Limitations of some methods of porphyrin isomer analysis

It can be seen from these simplified examples how mixtures of isomeric porphyrins can be obtained from a single pyrrole precursor, and how series III and IV porphyrins can predominate. It can also be confidently predicted that the exclusive formation of uroporphyrin III from porphobilinogen is not possible by chemical means. In the light of this, we have re-examined the uroporphyrin obtained by heating porphobilinogen in acid solution, which in most respects resembles uroporphyrin III. The results which are described in the experimental part show that no single method of isomer analysis is infallible, although a definite decision is usually possible when a combination of methods is used.

It has been established by Falk & Benson (private communication) that chromatography according to Chu *et al.* (1951) can only separate coproporphyrins I and II from coproporphyrins III and IV, but is unable to distinguish between the individual constituents of each pair. It appears from the present work that the same limitation applies to the Falk & Benson (1953) method for uroporphyrins. A more direct test of this point is not possible until pure uroporphyrins II and IV become available. Previous experimental data should be reconsidered with this reservation. The melting points of porphyrin esters are not reliable tests of purity; there are a number of examples in the literature of mixtures of different porphyrins (not even isomers) which melt within a degree of the melting point of one constituent alone. Uroporphyrin esters which melt at about 260° are a further example.

Consideration of the mechanism of the conversion of porphobilinogen into uroporphyrins suggested that the composition of the isomeric mixture might vary with the pH of the solution. Table 2 summarizes the results of experiments which confirmed this idea. The fact that (according to chromatograms) about half the uroporphyrin formed in alkaline solution consisted of uroporphyrin I (and/or II), whereas that formed at pH 6·5 consisted entirely of uroporphyrin III (and/or IV) emphasizes the artificial nature of Waldenström porphyrin. It may be significant that at the Minnesota School, 1% of Na_2CO_3 is sometimes used as a preservative for porphyria urine (cf. Schwartz, Zieve & Watson, 1951), for these were the conditions of Expt. *A* in Table 2. Scarcely any conversion into porphyrin took place when a solution of porphobilinogen in dilute hydrochloric acid was kept at room temperature (*D*, Table 2), although a high yield was obtained from a similar solution at 100°. Evidently the higher temperature is necessary for the decomposition of the salt of porphobilinogen, and at room temperature it was protected in this form. The violet pigment which slowly appeared may have been the result of oxidation.

Porphobilinogen in the biosynthesis of porphyrins

Numerous schemes for the biosynthesis of porphyrins have been put forward in the past. None was satisfactory as in the main they endeavoured to account for the production of I and III series porphyrins on purely chemical grounds. They usually involved as a final stage the condensation between two unlike dipyrrylmethenes which were arbitrarily forbidden to react with themselves. It was always assumed that the only position in a pyrrole at which substitution could take place was a 'free' position (i.e. one bearing a hydrogen atom), and thus any attempt to fit porphobilinogen into such a scheme would also require the presence of '*iso*porphobilinogen' (VII). No evidence has been found for the existence of an isomer of porphobilinogen in biological material; moreover, the foregoing discussion shows this to be unnecessary.

It has been shown that porphyrin formation from porphobilinogen in a chicken red cell haemolysate is an enzymic process, since porphobilinogen was recovered unchanged from a control experiment in which the haemolysate had been boiled (Falk, Dresel & Rimington, 1953). The isomeric composition of the porphyrins elaborated *in vivo* is the result of enzymically directed catalysis and need not be the same as that resulting from *in vitro* transformations, although the fundamental processes involved are presumably similar.

SUMMARY

1. Porphobilinogen has been shown to be 2-aminomethyl-4-2′-carboxyethyl-3-carboxymethylpyrrole (I)

2. 'Waldenström porphyrin' is an artifact derived from porphobilinogen by chemical means. It is a mixture of uroporphyrins, the composition of which varies according to the conditions under which it is formed.

3. The mechanism of porphyrin formation *in vitro* from such pyrroles as porphobilinogen is discussed. It is concluded that uroporphyrin III cannot be the sole product.

4. A mixture of isomeric uroporphyrins has been found, which behaved exactly as uroporphyrin III on kerosene–dioxan chromatography. It appears likely that this method can only separate uroporphyrins I and II from uroporphyrins III and IV.

5. No evidence has been found for the existence of an isomer of porphobilinogen.

6. The participation of porphobilinogen in porphyrin biosynthesis is briefly considered.

We are most grateful to all those who have provided us with porphyria urines. The expert technical assistance of Mr F. Grover in the isolation of porphobilinogen was a vital part of the work. The infrared data were kindly provided by Dr L. A. Duncanson, Imperial Chemical Industries Ltd.; we wish also to thank Dr S. F. MacDonald for gifts of chemicals and for the benefit of discussion. The porphyrin chromatography was kindly carried out by Miss A. Benson.

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The Inhibition of Growth and Cozymase Synthesis in Bacteria by Halogen-substituted Nicotinic Acids

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Among the non-metallic elements which form single covalent bonds, fluorine most nearly approaches hydrogen in atomic weight and size. The effect of the substitution of fluorine for hydrogen in molecules of essential metabolites is therefore of interest. This is especially so since it has been shown that monofluoroacetate is transformed into fluorocitrate which in its turn inhibits aconitase and thus blocks the tricarboxylic acid cycle (Peters, 1952). Fluorotyrosine and fluorophenylalanine have been shown to act as antagonists to tyrosine and phenylalanine in Neurospora (Mitchell & Nieman, 1947). More recently, Halvorson & Speigelman (1952) have shown that these two fluoro analogues inhibit adaptation in bacteria. The halogen-substituted nicotinic acids examined in this paper were synthesized for test as therapeutic agents (Minor, Hawkins, Vanderverf & Roe, 1949; Hawkins & Roe, 1949). One of them, namely 5-fluoronicotinic acid was shown to inhibit bacterial growth. It seemed likely that this inhibition was due to the inhibition of cozymase synthesis. This idea has been tested by growth tests as well as by studies of the effect of the analogues on cozymase synthesis in washed suspensions of bacteria as described in a previous paper (Hughes & Williamson, 1952*a*). The anticipated inhibition of cozymase synthesis by 5-fluoronicotinic acid has been found in three species of bacteria, but it has not been possible to establish definitely the precise point at which the synthesis is blocked.

EXPERIMENTAL

Organisms

Lactobacillus arabinosus 17–5 was maintained by daily subculture on the enzymic casein hydrolysate medium described by Nossal (1951). *Staphylococcus aureus*, National Collection of Type Cultures (N.C.T.C.) Strain 7663, *Bacterium coli*, N.C.T.C. Strain 86 and *Proteus vulgaris* I (a locally isolated strain) were maintained by subculture on agar slopes containing 4% yeast extract and 1% glucose.

Growth tests

The effect of nicotinic acid and its derivatives on the growth of *Lb. arabinosus* was studied in the supplemented casein hydrolysate medium of Barton-Wright (1946). Double-strength medium for *Staph. aureus* was made from stock solutions (Barton-Wright, 1946) as follows: hydrolysed casein, 50 ml.; inorganic salts *A* and *B*, 5 ml.; cystine, 50 ml.; tryptophan, 50 ml.; pyridoxine, 1·0 ml.; riboflavin, 1·0 ml.; biotin, 4·0 ml.; thiamine, 1·0 ml.; glucose, 10 g. were dissolved in 500 ml. of water and the pH was adjusted to 7·6 with NaOH. The medium for *Prot. vulgaris* and *Bact. coli* was prepared as follows: KH_2PO_4, 4·5 g.; NH_4Cl, 1·0 g.; $(NH_4)_2SO_4$, 1·0 g.; salts *B*, 5·0 ml.; hydrolysed casein, 10 ml.; pyridoxine, folic acid, pantothenic acid, thiamine, all 1·0 ml.; biotin, 4·0 ml.; glucose, 10 g. or lactic acid 2·5 g. These were dissolved in 800 ml. of water, the pH was adjusted to 7·6 with $NaOH_2$ and then simmered for 10 min., cooled and filtered. All the media were stored at 2° over $CHCl_3$ and sterilized at 10 lb./sq.in. for 10 min. before test. In growth tests with *Lb. arabinosus* and *Staph. aureus* the medium (2·0 ml.) and the solutions under test (2·0 ml.) were mixed in $6 \times \frac{1}{2}$ in. test tubes which were covered with aluminium caps (Oxo Ltd., London) and then sterilized. Cozymase was sterilized by filtration through a Seitz filter and added to the sterilized medium aseptically; no significant amounts of cozymase were absorbed by the filter pad. After sterilization the test tubes were inoculated with one drop of a washed suspension of the cells which had been diluted to contain approximately 10 000 cells per ml. In tests with *Prot. vulgaris* and *Bact. coli* the final volume of medium was 10 ml. and 50 ml. conical flasks were used in the place of test tubes. The temperature of incubation was 37° except with *Bact. coli*, which was incubated at 30°. Growth was measured turbidimetrically in a EEL Nephelometer (Evans Electro Selenium Ltd.) when test tubes were used, or by eye when flasks were used. *Lb. arabinosus*, which adhered to the side of the test tube during the first hours of growth, was evenly suspended by blowing a sterile air stream through the medium before turbidimetric measurements.

Preparation and handling of washed suspensions

To study the effect of the inhibitors on the catalytic function and synthesis of cozymase, deficient cells were produced as follows: *Lb. arabinosus* 17–5 was grown for 22–44 hr. on the supplemented casein hydrolysate medium (Barton-Wright, 1946) containing 2×10^{-7} M nicotonic acid (see Hughes & Williamson, 1952*a*). *Staph. aureus*, 6773, was grown for 18–20 hr. in Roux bottles on the solid medium described by Krebs (1937); these cells were deficient in thiamine as well as cozymase (see p. 491), but it was found that the addition of thiamine either to the medium or to washed suspensions made no difference to the rate of cozymase synthesis. *Prot. vulgaris* was grown on the vitamin and casein supplemented salt medium already described to which had been added 2×10^{-7} M nicotinic acid. After

growth, the *Lb. arabinosus* and *Prot. vulgaris* were collected by centrifuging and washed once in 0·9% NaCl. *Staph. aureus* was washed from the surface of the agar with 0·9% NaCl, the washings strained through glass wool, the cells collected by centrifuging and washed once again. After washing, the organisms were suspended in Krebs & Henseleit (1932) saline to give suspensions containing 2–10 mg. dry wt./ml. Dry weights were estimated by heating samples at 110° to constant weight and subtracting the dry weight of an equal volume of saline.

The effect of nicotinic acid and its analogues on glycolysis by washed suspensions was followed manometrically in the Warburg apparatus by measuring the evolution of CO_2 from a bicarbonate buffer as described by Hughes & Williamson (1952*a*). O_2 uptake by *Prot. vulgaris* was also followed manometrically; each Warburg cup was filled with 5–10 mg. dry wt. of cells suspended in 1·8 ml. Krebs & Henseleit (1932) saline containing 0·025 M glucose in the main compartment. The centre well contained 0·2 ml. of 2 M-KOH and a folded paper to absorb CO_2. When manometric data were not required cozymase synthesis was carried out in inverted 'Y' tubes (Hughes & Williamson, 1952*b*). The reaction was stopped by placing the vessel in an ice–salt freezing mixture. The cells were collected and washed by centrifuging as described previously (Hughes & Williamson, 1952*a*).

Nicotinic acid derivatives

The nicotinic acid, nicotinamide and cozymase were the specimens described in a previous paper (Hughes & Williamson, 1952*a*). The halogen-substituted nicotinic acids, and a small sample of 5-fluoronicotinamide synthesized according to Hawkins & Roe (1949), were kindly given by Dr K. Rohrman, Ely Lilly International Corporation, Indianapolis, U.S.A. The 2-, 5- and 6-monofluoronicotinic acid (2-FNA, 5-FNA, 6-FNA) showed no contamination with nicotinic acid on paper chromatography (see below), and were therefore not purified further. The 5-chloro- and 5-bromo-nicotinic acids (5-ClNa, 5-BrNA) had been found to be contaminated with nicotinic acid and were purified as follows: 324 mg. of the crude 5-BrNA were dissolved by warming in 20 ml. of the *n*-butanol phase of the butanol acetic solvent of Partridge & Westall (1948). The butanol solution was transferred to a powdered cellulose column (1·5 cm. wide by 50 cm. long) which had been previously washed with the butanol solvent until no material absorbing at 200 mμ. was detected in the eluate. The chromatogram was developed with the butanol:acetic solvent and 5 ml. fractions were collected in an automatic fraction collector. From each fourth fraction 0·1 ml. was taken and tested with CNBr and NaOH for 5-BrNA. The bulk of the substance was in fractions 18–26 and these were therefore pooled, and evaporated to dryness at 60°, *in vacuo*. The residue was recrystallized twice from an ethanol:water mixture. The crystals were finally washed with water, dried over P_2O_5 and weighed 165 mg.; no nicotinic acid could be detected in this material. The 5-ClNA was purified similarly.

Identification of nicotinic acid and derivatives

The reaction with CNBr and NaOH (Hughes & Williamson, 1953) and ultraviolet absorption were used to identify nicotinic acid derivatives on chromatograms. After treatment with CNBr and NaOH the absorption spectra of nicotinic acids substituted at carbon-atom 5 were similar to that of nicotinic acid; the absorption maxima were between 370 and 380 mμ. The molecular extinction coefficients measured at 372 mμ. (ϵ) were, however, lower than those of nicotinic acid (Table 1, column 2); 2-FNA and 6-FNA did not react with CNBr. Solutions of nicotinic acid and the halogeno derivatives in N-NCl or N-NaOH had absorption spectra between 230 and 300 mμ., similar to other pyridine derivatives; the maxima were between 260 and 280 mμ. (Table 1).

The detection of nicotinic acid in halogeno nicotinic acids by paper chromatography

Aqueous solutions of the halogeno acids (50 μl. of 0·1 M) were placed on Whatman no. 1 paper strips with an Agla syringe and the spots dried in a warm air stream. The chromatograms were developed in a descending butanol: acetic mixture (Partridge & Westall, 1948) for 16 hr., dried at 60–80° in an air stream and photographed on to reflex paper in ultraviolet light as described by Markham & Smith (1949). Spots containing 5 μl. of 0·03 M nicotinic acid were just visible by this method. The spots were approximately circular with a diameter of 2–3 cm. The R_F value of nicotinic acid (0·72) was sufficiently different from that of the halogeno nicotinic acids (0·8–0·9) to make a good separation possible. To isolate the nicotinic acid derivatives from the paper, the positions of the spots were marked with pencil and the paper corresponding to the spots was cut out. Each piece of paper was then extracted with 3 ml. water by heating for 10 min. in a boiling water bath. The growth-promoting activity of the extracts was estimated by microbiological assay. Two separate growth-promoting fractions were obtained from 5-BrNA nicotinic acid, one whose R_F corresponded to that of nicotinic acid (0·78) and the other whose R_F was less than that of nicotinic acid (0·38), only one spot whose R_F corresponded to that of nicotinic acid was obtained in chromatograms of crude 5-ClNA. In each case the main absorbing spot on the chromatogram, which was assumed to be that of the halogen-substituted nicotinic

Table 1. *Spectrophotometric data of nicotinic acid and halogeno nicotinic acids*

Compound	Absorbancy after reaction with CNBr	Absorbancy of solution in 0·1 N-HCl		Absorbancy of solution in 0·1 N-NaOH	
	$\epsilon_{372\ m\mu.}$ ($\times 10^{-4}$)	$E_{max.}$ (mμ.)	$\epsilon_{max.}$ ($\times 10^{-3}$)	$E_{max.}$ (mμ.)	$\epsilon_{max.}$ ($\times 10^{-3}$)
Nicotinic acid	3·02	260	4·36	260	3·10
2-Fluoronicotinic acid	No reaction	266	4·42	264	5·86
5-Fluoronicotinic acid	1·44	270	4·84	270	4·00
6-Fluoronicotinic acid	No reaction	262	3·70	263	3·70
5-Chloronicotinic acid	1·62	275	4·04	270	3·96
5-Bromonicotinic acid	1·25	280	3·32	270	5·36

acids, was without growth-promoting activity. As judged by the growth-promoting activity of the spots from the chromatograms, the amount of nicotinic acid in the crude 5-ClNA was 0·004% (w/w), while the combined spots from the crude 5-BrNA corresponded to a contamination of 0·025% (w/w). This degree of contamination was sufficient to explain the apparent growth-promoting activity found in the crude compounds.

Spectrophotometric determination of cozymase

This method consists of measuring the increase in optical density of extracts of the cells at 340 mμ. after reduction with crystalline alcohol dehydrogenase and ethanol as described by Racker (1949). Spectrophotometric measurements were made in the micro-cells of a Spekker Absorptiometer (1 cm. light path, 2 mm. wide and 8 mm. deep) fitted to a micro-attachment constructed as described by Lowry & Bessey (1946), but adapted to a Unicam Spectrophotometer (Hughes, 1951). The cozymase was extracted by heating the cells suspended in 0·5 ml. of water for 20 min. at 75°, the solid residue was separated by centrifuging and 0·3 ml. of the clear supernatant pipetted into a $2 \times \frac{3}{8}$ in. test tube. A solution (0·04 ml.) containing 0·5 N sodium pyrophosphate and 0·1 M ethanol was added, and the mixture was then transferred with a dropping pipette to the absorptiometer cell, and the optical density at 340 mμ. ($E^{1\,cm.}_{340\,m\mu}$) measured.

Table 2. *Effect of halogeno nicotinic acids upon the growth of* Lb. arabinosus *stimulated by varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was $2{\cdot}5 \times 10^{-3}$ M in all cases.

Inhibitor	Nicotinic acid ($M \times 10^5$)	Growth Nephelometer* readings after 16 hr.	23 hr.	72 hr.
None	None	5	6	7
	250	19	56	82
	25	20	56	74
	2·5	22	52	72
	0·25	14	48	68
5-Fluoronicotinic acid	250	6	20	60
	25	4	10	58
	2·5	5	5	10
	0·25	6	6	6
2-Fluoronicotinic acid	250	28	51	84
	25	10	44	78
	2·5	16	41	76
	0·25	6	16	62
6-Fluoronicotinic acid	250	22	54	78
	25	14	52	78
	2·5	20	52	80
	0·25	10	36	62
5-Chloronicotinic acid	250	14	50	66
	25	14	52	66
	2·5	16	56	72
	0·25	8	52	66
5-Bromonicotinic acid	250	19	54	82
	25	16	54	74
	2·5	20	56	72
	0·25	9	52	68

* Nephelometer reading of 100 is equal to 2·8–3·0 mg. dry wt. bacteria/ml.

The solution was then returned to the test tube, and 0·01 ml. of a solution of alcohol dehydrogenase was added. After a thorough mixing, $E^{1\,cm.}_{340\,m\mu}$ was measured when it had reached equilibrium (3–5 min.). The concentration of enzyme was such that 40 μm-mole of cozymase was reduced in approximately 2 min. The cozymase content of the sample was calculated from the difference between the two readings, using the value for the molecular extinction coefficient ($\epsilon = 6{\cdot}22 \times 10^3$) of Horecker & Kornberg (1948). Parallel estimations on extracts of *Lb. arabinosus* and *Staph. aureus* indicated that the spectrophotometric method gave values, some 10–15% lower than the fermentation method used previously (Hughes & Williamson, 1952*a*). This suggests that either nicotinamide riboside, or reduced cozymase are also present in the cells.

RESULTS

Growth tests

Effect of halogeno nicotinic acids on the growth of Lb. arabinosus. The five halogeno nicotinic acids inhibited to varying degrees the growth of *Lb. arabinosus* (Table 2). In all cases the magnitude of the inhibition was dependent not only on the concentration of the inhibitor but also the concentration of the nicotinic acid: i.e. the inhibition was competitive over the range tested (10^{-3} to 10^{-5} M) (cf. Tables 2 and 3). Differences between the inhibitors were most marked at the early stages of growth. As judged by inhibition indices the order of effectiveness was as follows: 5-FNa > 5-ClNA > 5-BrNa > 2-FNA = 6-FNA. After 5 days of incubation,

Table 3. *Effect of 5-fluoronicotinic acid upon the growth of* Lb. arabinosus *stimulated by nicotinic acid, nicotinamide or cozymase*

For conditions see text.

Growth factor added ($M \times 10^5$)	5-Fluoronicotinic acid (conc.)	Growth Nephelometer readings after 16 hr.	23 hr.	60 hr.
Nicotinic acid				
5·0	None	46	51	87
0·5	None	42	57	56
0·05	None	38	47	50
5·0	$M \times 10^{-3}$	45	58	82
0·5	$M \times 10^{-3}$	20	86	73
0·05	$M \times 10^{-3}$	4	5	40
Nicotinamide				
5·0	None	49	88	88
0·5	None	42	59	88
0·05	None	20	26	25
5·0	$M \times 10^{-3}$	44	96	82
0·5	$M \times 10^{-3}$	12	56	23
0·05	$M \times 10^{-3}$	5	5	40
Cozymase				
5·0	None	48	78	88
0·5	None	34	38	88
0·05	None	11	14	17
5·0	$M \times 10^{-3}$	29	90	91
0·5	$M \times 10^{-3}$	8	55	87
0·05	$M \times 10^{-3}$	5	5	9

maximal growth was found in all tubes except where 5-FNA was present. Thus 5-FNA is the most effective antagonist of nicotinic acid. As cozymase synthesis is the only known reaction utilizing nicotinic acid in this organism (Hughes & Williamson, 1952*a*) and as the inhibition is competitive, it may be inferred that the conversion of nicotinic acid into cozymase is inhibited, and the question arises as to which step is affected. To decide this, the effect of nicotinamide and cozymase in reversing the inhibition by 5-FNA was examined. Their effect in overcoming the inhibition by 5-FNA was no greater than that of nicotinic acid (Table 3) despite the fact that both are readily available for growth (Ellinger & Abdel-Kader, 1949). The reversal was also competitive and this might be explained by the assumption that it was the function of cozymase that was inhibited rather than its synthesis. That this is not so is shown by the experiments with washed suspensions described below.

Effect of halogeno nicotinic acids on the growth of Staph. aureus. The early growth of *Staph. aureus* was also inhibited by the halogen-substituted nicotinic acids (Table 4); the magnitude of the inhibition was dependent not only on the concentration of the inhibitor but also on the concentration of nicotinic acid (Table 4). Inhibition indices after 18 hr. growth indicated that as in *Lb. arabinosus*, 5-FNA was the most effective antagonist of nicotinic acid. In contrast to *Lb. arabinosus* and *Prot. vulgaris*, the inhibition was not completely reversed in *Staph. aureus* after 4 days' growth, even in the highest concentrations of nicotinic acid (Table 4). This effect was most marked in the case of 5-FNA acid. As judged by their effects on the early growth, nicotinamide and cozymase were less readily available for growth than nicotinic acid; likewise they were both less effective in reversing the inhibition caused by 5-FNA.

Effect of halogeno nicotinic acids on the growth of Prot. vulgaris. All of the halogeno nicotinic acids were less effective in inhibiting the growth of *Prot. vulgaris* than they were for the other two organisms (Table 5), but again 5-FNA acid was the most effective. The inhibition was competitive and was completely reversed in the higher concentrations of nicotinic acid ($2{\cdot}5 \times 10^{-6}$ M) and 5-FNA (10^{-3} M). Nicotinamide, although readily available for growth, was no more effective in reversing the inhibition than was nicotinic acid. Cozymase was less readily available for growth and less effective than the other two growth factors in reversing the inhibition.

Effect of halogeno nicotinic acids on the growth of Esch. coli. Of the halogeno nicotinic acids, only 5-FNA inhibited the growth of *Esch. coli*. The inhibition was marked in the early growth (16–18 hr.) and was competitively reversed by nicotinic acid. Later

Table 4. *Effect of halogeno nicotinic acids upon the growth of* Staph. aureus *stimulated by varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was $2{\cdot}5 \times 10^{-3}$ M in all cases.

Inhibitor	Nicotinic acid (M × 10^8)	Growth Nephelometer readings after 16 hr.	23 hr.	74 hr.
None	None	8	9	8
	250	34	48	86
	25	34	44	86
	2·5	36	48	84
	0·25	12	15	88
5-Fluoronicotinic acid	250	12	21	36
	25	10	18	36
	2·5	10	10	40
	0·25	9	9	10
2-Fluoronicotinic acid	250	22	37	56
	25	18	47	69
	2·5	20	48	70
	0·25	12	12	35
6-Fluoronicotinic acid	250	18	26	40
	25	18	26	45
	2·5	14	28	45
	0·25	10	10	16
5-Chloronicotinic acid	250	20	26	42
	25	18	28	44
	2·5	20	28	44
	0·25	11	10	40
5-Bromonicotinic acid	250	21	28	42
	25	20	26	42
	2·5	16	23	43
	0·25	12	10	43

Table 5. *Effect of halogeno nicotinic acids on the growth of* Proteus vulgaris *at varying concentrations of nicotinic acid*

For conditions see text. The concn. of inhibitor was 10^{-3} M in all cases.

Inhibitor	Nicotinic acid concn. (M × 10^8)	Growth Nephelometer readings after $15\frac{1}{2}$ hr.	24 hr.
None	None	7	7
	10	60	91
	1·0	60	96
	0·1	37	55
5-Fluoronicotinic acid	10	63	93
	1·0	24	63
	0·1	6	17
2-Fluoronicotinic acid	10	60	96
	1·0	47	79
	0·1	9	91
6-Fluoronicotinic acid	10	60	90
	1·0	45	90
	0·1	12	50
5-Chloronicotinic acid	10	63	87
	1·0	34	83
	0·1	31	67
5-Bromonicotinic acid	10	64	90
	1·0	60	93
	0·1	41	85

(24–30 hr.) the cells grew out fully in the highest concentrations of the inhibitor and without the addition of nicotinic acid. This is presumably because this organism, unlike the previous three, can synthesize its own nicotinic acid (Table 6).

Experiments with washed suspensions

Effect of halogeno nicotinic acids on glycolysis in Lb. arabinosus. Glycolysis in washed suspensions of *Lb. arabinosus* was not inhibited by any of the halogeno nicotinic acids in concentrations up to 5×10^{-3} M. In contrast to this the acceleration of glycolysis which occurs on the addition of nicotinic acid, nicotinamide and nicotinamide riboside was inhibited by 5-FNA (Fig. 1), but not by the other analogues. The magnitude of the inhibition was dependent not only on the concentration of the 5-FNA added but also on the concentration of the added nicotinic acid or its derivative; i.e. the inhibition was competitive. The acceleration of glycolysis by cozymase and nicotinamide ribonucleotide was not inhibited by 5-FNA. These results suggest that it is the synthesis and not the uptake and function of cozymase that is inhibited. The inactivity of the other halogen analogues was not unexpected because their effect on growth is transitory and they are at least 100-fold less efficient antagonists of nicotinic acid than the 5-fluoro analogue.

Effect of halogeno nicotinic acids on cozymase synthesis and uptake in Lb. arabinosus. The synthesis of cozymase from nicotinic acid by washed suspensions of *Lb. arabinosus* was inhibited to some extent by each of the five halogeno nicotinic acids (Table 7). In a concentration of nicotinic acid which permitted maximal rate of synthesis (5×10^{-5} M),

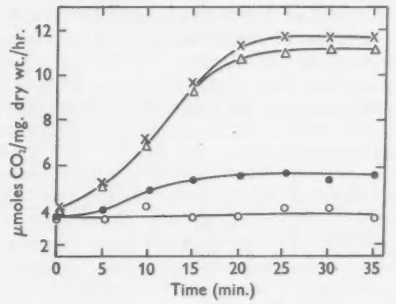

Fig. 1. Effect of 5-fluoronicotinic acid on the acceleration of glycolysis by nicotinic acid and cozymase in *Lb. arabinosus*. For conditions see Experimental, page 486. ○—○, no nicotinic acid; ×—×, nicotinic acid (5×10^{-5} M); ●—●, nicotinic acid (5×10^{-5} M) and 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-4}$ M); △—△, cozymase (5×10^{-5} M) and 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-4}$ M).

Table 6. *Effect of 5-fluoronicotinic acid on the growth of* Esch. coli

Growth conditions were as described in the text. + + + + to + Indicates gradation of growth from full growth to just detectable growth. 0 indicates no growth.

Addition to the medium	Growth after 16 hr.	20 hr.	24 hr.	36 hr.
None	+	+ +	+ + +	+ + + +
5-FNA (5×10^{-3} M)	0	0	+	+ + + +
5-FNA (5×10^{-3} M) and nicotinic acid (10^{-6} M)	+	+ +	+ +	+ + + +
5-FNA (5×10^{-3} M) and nicotinic acid (5×10^{-7} M)	0	+	+	+ + + +
5-FNA (5×10^{-3} M) and nicotinic acid (10^{-8} M)	?	?	+	+ + + +
Nicotinic acid (10^{-6} M)	+	+ +	+ + +	+ + + +

Table 7. *Inhibition of cozymase synthesis by halogeno nicotinic acids in* Lb. arabinosus *and* Staph. aureus

Washed cells were incubated in the glucose–saline mixture containing nicotinic acid (5×10^{-5} M) and inhibitor. After 15 min. incubation at 37° cozymase was estimated as described.

Inhibitor	Inhibitor concn. (M × 10^3)	*Lb. arabinosus* Cozymase synthesized (μm-mole)	*Lb. arabinosus* Inhibition (%)	*Staph. aureus* Cozymase synthesized (μm-mole)	*Staph. aureus* Inhibition (%)
None	—	6·6	—	10·2	—
5-FNA	2·5	2·6	70	0	100
	0·25	3·6	45	30	100
	0·05	4·8	26	30	24
2-FNA	2·5	4·6	30	7·1	30
	0·25	6·5	None	10·6	None
6-FNA	2·5	5·7	14	6·5	37
	0·25	6·6	None	10·3	None
5-ClNA	2·5	5·2	21	10·5	None
	0·25	6·6	None	10·8	None
5-BrNA	2·5	5·3	21	6·4	33
	0·25	6·6	None	10·2	None

inhibition (26 %) was found with 5-FNA (5×10^{-5} M), whilst no inhibition was found under these conditions with the other analogues. Thus, as would be expected from the previous experiments on growth and glycolysis, the 5-fluoro analogue is by far the most effective inhibitor of cozymase synthesis. The magnitude of the inhibition of the synthesis from nicotinamide and nicotinamide riboside by 5-FNA was the same as that from nicotinic acid (Table 8). The inhibition of the synthesis from nicotinamide ribonucleotide was variable, in some experiments being the same but in others only 30–40 % of that from nicotinic acid. The uptake of cozymase was not usually inhibited when cozymase and inhibitor were added simultaneously (Table 8). In concentrations of nicotinic acid from 10^{-3} to 10^{-6} M the magnitude of the inhibition of cozymase synthesis by 5-FNA (2×10^{-3} to 10^{-5} M) was greatest in the lowest concentrations of nicotinic acid and least in the highest concentration. The inhibition was of a competitive type, although in most batches of cells the reversal of the inhibition was never complete in the highest concentration of nicotinic acid. Similar results were found with nicotinamide over the same range of concentrations (Table 9), and with nicotinamide riboside over a smaller range of inhibitor (5×10^{-4} to 10^{-5} M) and metabolite (10^{-5} to 10^{-6} M) concentrations tested. In the foregoing experiments the inhibitor and nicotinic acid derivative were added simultaneously. When the inhibitor was added before the metabolite, the magnitude of the inhibition was no longer dependent on the concentration of the metabolite but mainly on the length of time of the pre-incubation with the inhibitor (Table 10). Under these conditions the effectiveness of 5-FNA, when judged by the lowest concentration at which inhibition was found, was increased some 100- to 1000-fold by the prior incubation (Table 11). The nature of the inhibition also appeared to change and was no longer competitive when the inhibitor was added before the metabolite (Table 12). It is also noteworthy that in two of five experiments in which cozymase was added after a prior incubation of cells with 5-FNA, the uptake was inhibited (20–30 %), whereas no inhibition of uptake was found when cozymase and inhibitor were added simultaneously. If, after a pre-incubation of cells with inhibitor, the excess of inhibitor was removed

Table 8. *Effect of 5-fluoronicotinic acid on cozymase synthesis from nicotinic acid and its derivatives and the uptake of cozymase in* Lb. arabinosus

Washed cells (3·0 mg. dry wt.) were incubated in glucose–saline and with the additions indicated below. Cozymase was estimated after 20 min. incubation at 37°. The concn. of growth factor was $2{\cdot}5 \times 10^{-5}$ M in all cases.

Growth factor added	5-Fluoronicotinic acid concn. (M × 10^5)	Cozymase synthesized (μm-mole)
Nicotinic acid	None	8·5
	2·5	5·1
	0·25	7·4
Nicotinamide	None	9·2
	2·5	3·3
	0·25	5·0
Nicotinamide riboside	None	10·2
	2·5	3·3
	0·25	6·5
Nicotinamide ribonucleotide	None	10
	2·5	3·0
	0·25	6·5
Cozymase	None	5·0
	2·5	5·2
	0·25	5·0

Table 9. *The inhibition by 5-fluoronicotinic acid of cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide in washed suspensions of* Lb. arabinosus

Washed cells (9·5 mg. dry wt.) suspended in 1·0 ml. glucose–saline were added to 1·0 ml. water containing 5-fluoronicotinic acid (5×10^{-4} M) and nicotinic acid in one experiment and nicotinamide in another experiment. After 10 min. incubation at 37° cozymase was estimated in the cells as described in the text.

Nicotinic acid concn. (M × 10^5)	Cozymase synthesized (μm-mole) Without inhibitor	With inhibitor	Inhibition (%)
10	12·4	7·0	44
5	10·9	6·2	44
1·0	12·9	3·9	68
0·5	7·0	1·0	86
Nicotinamide concn. (M × 10^5)			
10	20	19·6	2
5	20·2	17·9	10
1·0	18·6	10·2	40
0·5	7·9	1·5	80

Table 10. *Effect of time of incubation with 5-fluoronicotinic acid on the inhibition of cozymase synthesis in* Lb. arabinosus

Washed cells (4 mg. dry wt.) were incubated in the usual glucose–saline mixture with 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-4}$ M final concn.). Nicotinic acid ($2{\cdot}5 \times 10^{-5}$ M) was then added and after another 10 min. the cozymase content of the cells was estimated.

Time of incubation (min.)	Cozymase content of cells (μm-mole) Without FNA	With FNA	Inhibition (%)
0	14	None	—
0	14	12	14
10	14	11	21
20	12	5	59
40	11	1·5	82

by washing the cells in saline, cozymase synthesis was still found to be inhibited without the addition of further inhibitor (Table 13). Thus, not only was the effect of 5-FNA as an inhibitor of cozymase synthesis increased by the prior incubation but it was also taken up and retained by the cells on washing. The uptake and retention of 5-FNA is dependent on the presence of a concomitant glycolytic reaction (Table 13), suggesting that energy is needed either for its passage into the cell or for the retention.

Table 11. *Effect of pre-incubation with varying concentrations of 5-fluoronicotinic acid on cozymase synthesis in washed suspensions of* Lb. arabinosus

Washed cells (11·5 mg. dry wt.) suspended in 1·0 ml. of glucose–saline mixture were added to tubes containing the 5-fluoronicotinic acid as indicated below. Set *A* contained nicotinic acid (5×10^{-5} M) and were incubated for 15 min. at 27°. Set *B* contained no nicotinic acid but after the first 15 min. nicotinic acid was added and incubation continued for a further 15 min. Cozymase was extracted and estimated as described in the text.

5-Fluoronicotinic acid concn. (M × 10^5)	Cozymase synthesized (μm-mole)		Inhibition (%)	
	A	*B*	*A*	*B*
250	9·7	9·0	52	59
25	15·2	12·2	25	43
2·5	20	15·8	2	26
0·25	21	15·8	0	26
None	20·2	21·4	—	—

Table 12. *Effect of pre-incubation of cells with 5-fluoronicotinic acid on cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide*

Washed cells of *Lb. arabinosus* (8·5 mg. dry wt.) were incubated at 37° for 10 min. in the usual glucose–saline mixture and with and without 5-fluoronicotinic acid (2×10^{-4} M). Nicotinic acid and nicotinamide were then added as indicated below and the incubation continued for a further 15 min. Cozymase was extracted and estimated as described in the text.

	Cozymase synthesized (μm-mole)		
Nicotinic acid concn. (M × 10^5)	Without 5-FNA	With 5-FNA	Inhibition (%)
10	13·8	5·9	56
5	11·8	5·4	54
1	12·7	5·5	56
0·5	9·8	5·0	49
Nicotinamide concn. (M × 10^5)			
10	15	5·9	60
5	15	5·4	63
1	13	5·8	55
0·5	10	5·4	46

Effect of halogeno nicotinic acids on glycolysis and cozymase synthesis in Staph. aureus. The addition of nicotinic acid (5×10^{-5} to 5×10^{-6} M) to glycolysing washed suspensions of *Staph. aureus* stimulated their rate of glycolysis by some 20–40% (Fig. 2). Similar concentrations of nicotinamide and cozymase were usually without effect. This result agrees with the findings of the growth tests in suggesting that in this organism nicotinamide and cozymase are not so readily available as nicotinic acid.

Table 13. *Effect of glucose on the inhibition of cozymase synthesis by 5-fluoronicotinic acid in* Lb. arabinosus

2·5 mg. dry wt. of cells were incubated with the additions shown below. They were then separated and washed twice in 0·9% saline and resuspended in 1·8 ml. of the usual glucose–saline mixture. After 5 min. incubation at 37° nicotinic acid (5×10^{-5} M final) was added and cozymase estimated after a further 15 min. incubation.

Time of first incubation (min.)	Addition	Cozymase content of cells (μm-mole)	Inhibition (%)
10	None	8·3	—
10	Glucose ($2·5 \times 10^{-2}$ M)	8·5	—
10	5-FNA ($2·5 \times 10^{-4}$ M)	8·1	None
10	Glucose ($2·5 \times 10^{-2}$ M) and 5-FNA ($2·5 \times 10^{-4}$ M)	4·1	49
30	None	8·9	—
30	Glucose ($2·5 \times 10^{-2}$ M)	7·1	—
30	5-FNA ($2·5 \times 10^{-4}$ M)	6·5	27
30	Glucose ($2·5 \times 10^{-2}$ M) and 5-FNA ($2·5 \times 10^{-4}$ M)	2·1	70

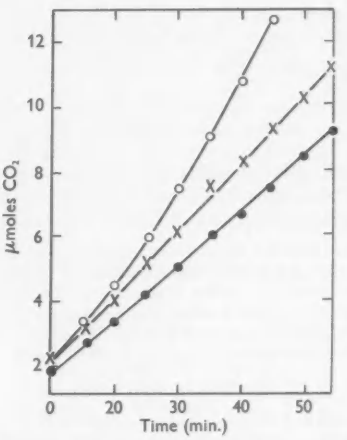

Fig. 2. Effect of 5-fluoronicotinic acid on the acceleration of glycolysis by nicotinic acid in *Staph. aureus*. For conditions see page 486. ●—●, no nicotinic acid; ×—×, nicotinic acid (5×10^{-5} M) and 5-fluoronicotinic acid (10^{-3} M); ○—○, nicotinic acid (5×10^{-5} M).

5-FNA inhibited the stimulation of glycolysis by nicotinic acid but did not inhibit glycolysis before the addition of nicotinic acid. Cozymase synthesis in *Staph. aureus* was, as in *Lb. arabinosus*, inhibited to some extent by all of the halogeno nicotinic acids (Table 6), but again 5-FNA was the most effective inhibitor. The inhibition of the synthesis from nicotinic acid by 5-FNA was competitive over the range tested (5×10^{-4} to 5×10^{-6} M). Synthesis of cozymase from nicotinamide was slower than from nicotinic acid but was also inhibited competitively (Table 14). The rate of uptake of cozymase was also slower than its synthesis from nicotinic acid but the uptake was not inhibited by 5-FNA. Prior incubation of the cells with the inhibitor increased the inhibitory effect of 5-FNA; this was independent of the presence of glucose in most batches of cells. The addition of fluoride (5×10^{-2} to 5×10^{-4} M) inhibited the uptake of 5-FNA as judged by the amount of cozymase synthesized after the excess of both inhibitors had been removed by washing (Table 15). It seems likely therefore that, as in *Lb. arabinosus*, 5-FNA is taken up and retained in cells of *Staph. aureus* and that this reaction depends on the presence of an energy-yielding reaction, such as glycolysis.

Table 14. *The inhibition by 5-fluoronicotinic acid of cozymase synthesis from varying concentrations of nicotinic acid and nicotinamide in washed suspensions of* Staph. aureus

Washed cells (7·5 mg.) suspended in glucose–saline (1·0 ml.) were added to 1·0 ml. aqueous solutions of nicotinic acid and nicotinamide as indicated below and 5-FNA (5×10^{-4} M). Cozymase content of the cells was estimated after 15 min. incubation at 37°.

	Cozymase synthesized (μm-mole)		
Nicotinic acid ($M \times 10^4$)	Without 5-FNA	With 5-FNA	Inhibition (%)
40	17·3	16·8	3
20	16·4	14·7	10
1	11·7	6·7	43
0·5	6·7	1·5	75
Nicotinamide ($M \times 10^4$)			
40	9·7	7·7	20
20	8·1	4·4	46
10	4·7	2·0	57
5	4·8	None	100

Table 15. *Effect of sodium fluoride on the inhibition of cozymase synthesis by 5-fluoronicotinic acid in* Staph. aureus

Washed cells (8·0 mg. dry wt.) were incubated 10 min. at 37° in the usual glucose–saline mixture and with 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-4}$ M final concn.). They were then washed twice in 0·9% saline and resuspended in 1·0 ml. glucose–saline and incubated with nicotinic acid (5×10^{-5} M). Cozymase was estimated in the cells after 20 min.

	Cozymase content of cells (μm-mole)		
Sodium fluoride ($M \times 10^3$)	Without 5-FNA	With 5-FNA	Inhibition (%)
None	14	None	100
25	17·5	17·0	3
2·5	13·5	4·1	70
0·25	11·6	2·0	90

Effect of 5-fluoronicotinic acid on oxygen uptake and cozymase synthesis in Prot. vulgaris. The oxygen uptake of washed suspension of *Prot. vulgaris* grown for 40–44 hr. in medium containing nicotinic acid (10^{-5} to 10^{-7} M) was followed manometrically. Oxygen uptake of the cells grown in the lower concentrations of nicotinic acid ($1{\cdot}0$ to $2{\cdot}0 \times 10^{-7}$ M) was stimulated (six- to eight-fold) by the addition of nicotinic acid, nicotinamide, nicotinamide ribonucleotide or cozymase (5 to $0{\cdot}5 \times 10^{-5}$ M): oxygen uptake in cells grown on the higher concentrations was not stimulated. 5-FNA did not inhibit oxygen uptake before the addition of nicotinic acid and its derivatives but did inhibit the stimulation by nicotinic acid, nicotinamide and nicotinamide ribonucleotide; the stimulating effect of cozymase was not inhibited (Fig. 3). The cozymase content of the cells (0·05 μm-mole/mg. dry wt.) was too low to be measured by the present methods (see also Jackson & Copping, 1952) and the nicotinamide nucleotide in the cells was therefore estimated by the acetone condensation method of Levitis, Robinson, Rosen, Huff & Perlzweig (1945). The apparent nicotinamide nucleotide content of the cells increased from 0·02 to 0·25 μm-mole/mg. dry wt. upon incubation with nicotinic acid and glucose; this increase was abolished by the addition of 5-FNA. It is likely

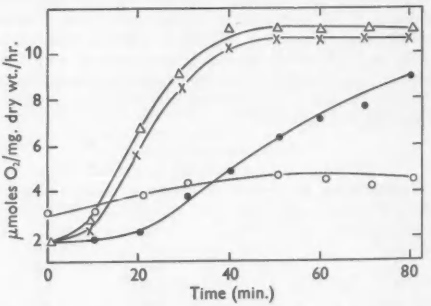

Fig. 3. Effect of 5-fluoronicotinic acid on the acceleration of oxygen uptake by nicotinic acid and cozymase in *Prot. vulgaris*. For conditions see page 486. ○—○, no nicotinic acid; ×—×, nicotinic acid (5×10^{-5} M); ●—●, nicotinic acid (5×10^{-5} M) and 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-3}$ M); △—△, cozymase (5×10^{-5} M) and 5-fluoronicotinic acid ($2{\cdot}5 \times 10^{-3}$ M).

therefore that in *Prot. vulgaris*, 5-FNA inhibits cozymase synthesis from nicotinic acid, nicotinamide and nicotinamide riboside but does not inhibit the uptake nor the function of cozymase.

DISCUSSION

The mechanism of the inhibition of cozymase synthesis in bacteria by 5-fluoronicotinic acid. Available evidence (Hughes & Williamson, 1952*a*) suggests the following route for cozymase synthesis:

nicotinic acid → nicotinamide (1)

nicotinamide → nicotinamide riboside (2)

nicotinamide riboside → nicotinamide ribonucleotide (3)

nicotinamide ribonucleotide → cozymase. (4)

From consideration of this scheme and according to the concepts of inhibition analysis (Shive & Roberts, 1946) it was expected that 5-FNA would inhibit at stage 1 competitively, and that synthesis from nicotinamide and its derivatives would not be markedly inhibited: any inhibition of synthesis from nicotinamide would be expected to be non-competitive. It was in fact found that the inhibition of synthesis from nicotinic acid was competitive over a reasonable range. Unexpectedly, synthesis from nicotinamide and nicotinamide riboside was inhibited to approximately the same extent as that from nicotinic acid and this inhibition was also competitive; synthesis from nicotinamide ribonucleotide was generally less markedly inhibited. The order of addition of inhibitor (5-FNA) and metabolite changed the competitive into non-competitive inhibitions and markedly increased their magnitude. Thus the action of the inhibitor (5-FNA) appears to be more complicated than the expected inhibition of the single stage leading to the formation of nicotinamide from nicotinic acid. It seems likely that either steps 1, 2 and 3 are all inhibited to some extent, or that step 3 alone rather than step 1 is inhibited. 5-FNA might bring about such inhibitions because (*a*) structural similarity between it and the presumed cozymase intermediates is sufficiently close to permit competition in each reaction, (*b*) 5-FNA is metabolically transformed into a more active inhibitor or inhibitors. That 5-FNA inhibits neither the deamidation of nicotinamide by *Lb. arabinosus* (Hughes & Williamson, 1953) nor cozymase-catalysed reactions suggests that mechanism (*a*) is unlikely. The changes which occur on incubation of inhibitor with glycolysing cells greatly favour the intervention of mechanism (*b*). Some other inhibitions are shown in Table 16 in which an analogue of an essential metabolite undergoes metabolic transformation. Of particular interest is the action of *iso*nicotinic acid hydrazide which undergoes an exchange reaction and is transformed into an analogue of cozymase. This does not depend on an energy-yielding reaction and thus differs from the other reactions but can be considered as forming a link between mechanism (*a*) and (*b*). The inhibitions may be considered as belonging to the type recently characterized by Peters (1952) as 'lethal synthesis', following studies of the inhibition of acetate oxidation by monofluoroacetate. The inhibition of cozymase synthesis by 5-FNA has the following points in common with inhibition by 'lethal synthesis':

(1) It is competitive when inhibitor and metabolite are added simultaneously, but non-competitive when the inhibitor is added before metabolite.

(2) The magnitude of the inhibition is increased by incubation of inhibitor with actively metabolizing cells.

(3) The inhibition takes place at several points in a metabolic sequence or at a point higher in a synthetic sequence than would be expected from structural similarity between inhibitor and metabolite.

(4) The inhibitor is taken up and retained by the cells. It seems feasible therefore that the inhibition of cozymase synthesis by 5-FNA might be a type of 'lethal synthesis' in which the inhibitor enters into the pathway of cozymase synthesis from nicotinic acid and blocks it at several points. If this were so it

Table 16. *Some synthetic transformations of analogues to form inhibitors of enzymic reactions ('lethal synthesis')*

Analogue	Inhibitor	Synthetic system	System inhibited	Reference
Deoxypyridoxine	Deoxypyridoxine phosphate	*Strep. faecalis*	Tyrosine decarboxylase	Umbreit & Waddell (1949)
Monofluoroacetate	Fluorocitrate	Mammalian liver	Aconitase	Peters (1952)
Ethionine	? Triethylcholine	Intact rats	Rat growth	Stekol & Weiss (1950)
*Iso*nicotinic acid hydrazide (INH)	INH analogue of cozymase	Neurospora DPNase	Mammalian DNPase	Zatman, Kaplan & Colowick (1953)
8-Azaguanine	? Nucleic acids	Intact rat tumour tissue and plant tissue	Rat growth, tumour growth and plant virus	Matthews (1953)

would be expected that derivatives of 5-fluoronicotinamide would appear in the cells during incubation with 5-FNA. Some attempts were therefore made to isolate such products (see Hughes, 1952*a*), and material tentatively identified as 5-fluoronicotinamide was isolated from large-scale experiments (5 g. dry wt. of cells of *Staph. aureus*). Full proof of the identity of this material as a fluorine-substituted pyridine derivative must await isolation in larger quantities. Because of the small amounts of inhibitor taken up by the cells (*c.* 1–2 μm-moles/mg. dry wt.) and because large amounts of deficient cells have to be used, such isolation presents many difficulties. Experiments have shown that in the more readily available baker's yeast, 5-FNA inhibits cozymase synthesis and is taken up and bound by the cells. It seems likely therefore that baker's yeast will be suitable material in which to study the metabolism of 5-FNA.

The significance of 'lethal synthesis' in the design of anti-bacterial agents. The advocacy by Fildes (1940) of a 'rational approach to chemotherapy' has led to the synthesis of a host of analogues of essential metabolites, but few have been of use as chemotherapeutic agents. This is probably because, as pointed out by Rydon (1949), the chemical approach has been based on pictorial likeness without knowledge of the enzymic mechanisms involved. In addition, the biological approach has been based on finding a 'nutritional weak point' in the bacteria and too often this has also been a 'weak point' in the host also. The simplicity of the antagonism between *p*-aminobenzoic acid and sulphonamide (Woods, 1940) has also led to a rigidity in interpretation along what Woolley (1952) has called the 'most favoured hypothesis', i.e. a competition between analogue and metabolite for a single enzyme site. However, Woolley (1952, p. 99), in discussing the effects of ethionine, has suggested the possibility that 'the action of a metabolite analogue... might thus not be due to a simple direct effect but rather to an accumulation of slight hindrances exerted along a chain of metabolic reactions'. This idea has been given further importance by the discoveries by Peters (1952) of the mechanism of fluoroacetate poisoning to which he applied the term 'lethal synthesis'. As shown in Table 16 some other inhibitors may also be considered as belonging to this type. It seems likely that potent inhibitors may be obtained when their design is based upon metabolites which normally enter into synthetic sequences, and if the inhibitors themselves will undergo some, if not all, of the synthetic transformations. For such inhibitory analogues to be successful chemotherapeutic agents they would by preference be based upon metabolites involved in synthetic sequences carried out by pathogens and not by the host organism. For instance, precursors of vitamin B_{12} might be considered as starting points since most bacteria synthesize their own supplies of this vitamin whilst higher animals cannot. Such considerations would tend to concentrate to a much greater extent than in the past, on the differences between synthetic ability of parasite and host. The choice of substituent groups in the design of such inhibitors must be governed by the experience gained in the last decade (see Woolley, 1952). It is well known, for instance, that the substitution of hydrogen by such groups as CH_3, NH_2, OH, Cl or Br often yields potent inhibitors (Albert, 1952). However, because of its small atomic size and because of the stability of the C—F bond, fluorine appears to be preferable to these groups when considering the design of compounds to take part in a 'lethal synthesis'. Additional evidence that fluoro analogues may be easily metabolized by bacteria and that the C—F bond remains intact is provided by studies on the oxidation of fluoronicotinic acids (Hughes, 1952*b*) and fluorobenzoic acids (Hughes, 1953).

SUMMARY

1. The growth of *Lactobacillus arabinosus*, *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli* is inhibited by the five halogeno nicotinic acids in the following order of effectiveness: 5-fluoronicotinic acid > 5-chloronicotinic acid > 5-bromonicotinic acid > 2- and 6-fluoronicotinic acids.

2. The inhibition is reversed competitively by nicotinic acid, nicotinamide and cozymase.

3. The acceleration of glycolysis by nicotinic acid in washed suspensions of *Lb. arabinosus* and *Staph. aureus* is inhibited by 5-fluoronicotinic acid; acceleration by cozymase in *Lb. arabinosus* is not inhibited. 5-Fluoronicotinic acid caused similar effects on the acceleration of oxygen uptake of *Prot. vulgaris* by nicotinic acid and its derivatives.

4. Cozymase synthesis from nicotinic acid, nicotinamide, nicotinamide riboside, and to a lesser degree nicotinamide ribonucleotide, is inhibited by 5-fluoronicotinic acid in *Lb. arabinosus*. Cozymase uptake is not inhibited. A similar inhibition of synthesis of cozymase from nicotinic acid, nicotinamide and nicotinamide ribonucleotide was found in *Staph. aureus*. The inhibition is competitive when inhibitor and metabolite are added simultaneously, but non-competitive when inhibitor is added before metabolite.

5. The magnitude of the inhibition of cozymase synthesis from nicotinic acid in *Lb. arabinosus* and *Staph. aureus* was increased at least tenfold by prior incubation of the cells with the inhibitor. During the incubation the inhibitor was taken up and bound by the cells.

6. The uptake of 5-fluoronicotinic acid is dependent on the presence of a concomitant glycolytic

reaction. It is suggested that 5-fluoronicotinic acid inhibits several steps in the synthesis of cozymase possibly by being metabolized along the synthetic route.

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The Regulation of Respiration during the Assimilation of Nitrogen in *Torulopsis utilis*

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In micro-organisms and in higher plants there is strong evidence that cellular respiration and the assimilation of inorganic nitrogen are closely interrelated metabolic processes. Some of the mechanisms underlying their linkage have been discussed by Yemm (1949), Burris & Wilson (1952), and Syrett (1953*b*). In order to investigate these interrelations more fully, experiments have recently been carried out with food yeast (*Torulopsis utilis*). This yeast has an outstanding capacity for the assimilation of nitrogen and synthesis of protein. Roine (1947) and Virtanen, Csaky & Rautanen (1949) have shown that pure cultures can be readily grown on a culture medium containing sucrose and minerals including ammonium salts, and then brought into a physiological condition in which they will rapidly utilize either ammonia or nitrate, by aeration for a few hours in a sucrose–mineral salt solution lacking in nitrogen. Their results indicate that glutamine, asparagine and alanine are the chief primary products of assimilation when such yeast is treated with a nitrogen source in the absence of external carbohydrate, and this clearly suggests a close parallel with the data now available for roots and other tissues of higher plants (e.g. Willis, 1950, 1951).

The experiments described in this paper were designed to investigate the interrelations of cellular respiration, the assimilation of nitrogen, and the metabolism of carbohydrates. For this purpose, pure cultures of the yeast were supplied with ammonium salts under controlled conditions; the

products of assimilation were determined together with the rates of respiration and of carbohydrate breakdown in the cells.

MATERIAL AND METHODS

Preparation of yeast suspensions

Food yeasts. Cultures of *Torulopsis utilis* (B.P. 60) and of *Torulopsis utilis* var. *major* (N.C.T.C. 6593) from the National Collection of Type Cultures were maintained on nitrate–sucrose agar. For experimental purposes the yeasts were grown on the complete culture medium (soln. *A*) modified from Virtanen *et al.* (1949) and with the following composition: sucrose 50 g., $(NH_4)_2HPO_4$ 3 g., $(NH_4)_2SO_4$ 4 g., K_2SO_4 1·5 g., $MgSO_4.7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution (Winzler, Burk & du Vigneaud, 1944: containing H_3BO_4 1 mg., $ZnSO_4$ 1 mg., $MnCl_2$ 1 mg., $FeCl_3$ 0·5 mg., $CuSO_4.5H_2O$ 0·1 mg. and KI 0·1 mg.), distilled water to 1 l. and the pH adjusted to 6·0–6·5 with HCl. Portions (125 ml.) of this medium were sterilized in 2 l. cylindrical culture flasks (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944), inoculated with a loopful of the yeast culture, and gently shaken on a 'Microid' flask shaker (Griffin and Tatlock Ltd.) in a constant-temperature room at 25° for 15–20 hr. When the culture became visibly turbid, the cotton-wool plugs were removed and the rate of shaking was increased to improve aeration for the next 4–5 hr. The yeast was then centrifuged off at 1500 *g* for 15 min. Total fresh weight was 1–2 g./flask.

Baker's yeast. Commercial pressed yeast (Distillers Co. Ltd.) was washed three times at the centrifuge with 20 times its weight of solution *C* (see below) at each washing.

Nitrogen-deficient cultures of yeast. The yeast, prepared as above, was resuspended in the N-deficient solution *B* modified from Virtanen *et al.* (1949), containing sucrose 50 g., KH_2PO_4 3 g., $MgSO_4.7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution, distilled water to 1 l. and with the pH adjusted to 7·2–7·5 with NaOH. A volume equal to that of the original culture was used and the suspension was divided into 125 ml. portions and shaken as before for 12–16 hr. at 25°. During this period the fresh weight of the yeast was approximately doubled. The culture was then centrifuged off and washed once by centrifuging in the mineral salt solution *C* modified from Virtanen *et al.* (1949). This solution contained KH_2PO_4 3 g., $MgSO_4.7H_2O$ 1 g., $CaCl_2$ 0·05 g., 10 ml. micronutrient solution, distilled water to 1 l., with the pH adjusted to 6·00 ± 0·05. The washed culture was resuspended in solution *C* to give 2 g. fresh weight yeast/100 ml. suspension.

Experimental procedures

Measurement of respiration. O_2 uptake was measured manometrically at 25° by the direct method of Warburg (Dixon, 1943). The N-deficient yeast suspension (3 ml.) was pipetted into the main compartment of each flask, with 0·5 ml. of 0·06 M-$(NH_4)_2HPO_4$, or 0·06 M-Na_2HPO_4, or of appropriate mixtures of the two, in the side bulb. The phosphate solutions were adjusted to pH 6·00 ± 0·05 with M-H_3PO_4. In experiments involving the use of 2:4-dinitrophenol appropriate amounts of this were included in the 0·5 ml. total volume in the side bulb. CO_2 output (and thus R.Q.) was occasionally determined on replicate samples by the direct method (Dixon, 1943). Bound CO_2 was estimated by the addition of 0·5 ml. 3 N-HCl from the side bulb, but the correction was small, never amounting to more than 2·5% of the total CO_2 produced in respiration.

Cultures for analysis. When large quantities of yeast suspension were required for analysis, samples were treated in the cylindrical culture flasks on the shaking machine at 25°, while a parallel experiment was performed in manometers to yield respiratory data. Each culture flask contained 90 ml. yeast suspension, and 15 ml. of the appropriate mixture of 0·06 M-$(NH_4)_2HPO_4$ and 0·06 M-Na_2HPO_4 at pH 6·0 was added after a suitable interval. The flasks were sampled after various periods of treatment, the contents of one flask being taken on each occasion. The suspension was washed into a centrifuge bottle with 20–30 ml. distilled water and centrifuged for 15 min. at 1500 *g*. The clear supernatant was decanted and made to 150 ml.; 5 ml. were then removed for pH determination by means of a glass electrode. The remainder of the culture solution was acidified with 5 ml. 4 N-H_2SO_4 and stored in a refrigerator for subsequent analysis. The sedimented yeast was weighed in the bottle and then chilled in an ice bath in preparation for analysis.

Analytical methods

Dry weight. Samples (1 ml.) of yeast suspension were dried at 98° in small weighing bottles until a constant value was reached (4–8 hr.). A blank, consisting of 1 ml. of the suspending solution, was dried alongside and used to correct the estimations.

Extraction of soluble constituents. The yeast, separated by centrifuging as described above, was chilled in the ice bath for 5 min. and treated with 2 ml. cold absolute ethanol for 15 min. Cold 0·2 N-H_2SO_4 (20 ml.) was then pipetted in, and the mixture was left in the ice bath for 1 hr. with shaking every 5 min. The yeast was then centrifuged (10 min. at 1500 *g*) and the extract decanted and saved for analysis. The yeast was re-extracted with 20 ml. 0·2 N-H_2SO_4, centrifuged, the second extract discarded, and the residue suspended in distilled water, made to 20 ml. and saved for analysis.

In this extraction procedure it was assumed that all soluble components came freely into solution, and the proportion extracted was the same as the proportion of total extractant recovered from the yeast. As the yeast contained approx. 80% moisture and was extracted with 2 ml. ethanol and 20 ml. H_2SO_4, the volume of total extractant used in ml. was $2 + 20 + 0{\cdot}8 \times$ (fresh wt. yeast in g.) + wt. solution held on centrifuge bottle (in g.), making a total of 23·8–24·2 ml. in most experiments. Estimations of total N and glutathione on serial extracts showed the errors caused by these assumptions to be less than 1% of the total amount extracted.

Ammonia. The free-ammonia content of culture solution, extracts, and of the yeast suspensions from the manometric experiments, were determined by the boric acid–HCl procedure of Conway (1950) in standard microdiffusion units. The solution under test (0·5 ml.) was treated with 0·5 ml. saturated KBO_2 and left to diffuse at 0–5° for 90–120 min. before titration.

Total nitrogen. Portions (2 ml.) of extracts or residues were heated with 0·5 g. catalyst (Yemm & Folkes, 1953) and 2 ml. conc. H_2SO_4 for 20 hr. When cool, the solution was diluted to 10 ml. for extract or 25 ml. for residue. Portions (1 ml.) were taken for ammonia estimation as above but were neutralized with 1 ml. 10 N-NaOH for extract or 1 ml. alkaline metaborate (equal parts of saturated KBO_2 and

7N-KOH) for residue. Diffusion was allowed to proceed for 18–20 hr. at room temperature.

Glutamic acid and glutamine. The free amino acid and the amide were estimated by means of the specific glutamic decarboxylase and glutaminase preparation of *Clostridium welchii* (S.R. 12) by the method developed by Krebs (1948). To 2 ml. extract was added 1 ml. acetate–cetavlon (0·45M acetate buffer pH 4·9 containing 0·15% cetyltrimethylammonium bromide) and 0·8N-NaOH (to adjust pH to 4·8–5·0). Part of this mixture (3 ml.) was used for the estimation of glutamic acid using 12·5 mg. of a freeze-dried enzyme preparation in 0·5 ml. 0·15M acetate buffer, pH 4·9. Glutamine is both deamidated and decarboxylated by the enzyme (Krebs, 1948) and the glutamine content was calculated from the free ammonia present at the end of the enzyme treatment. For this estimation, 0·5 ml. portions were taken for the Conway units and 0·5 ml. saturated KBO_2.

The above procedure could also be applied to the small volumes of yeast suspension used for the manometric measurement of respiration, but here a preliminary extraction was necessary. The suspension (2 ml.) was pipetted into a tube containing 0·5 ml. 5N-HCl, and 1 ml. acetate–cetavlon was added. The solution was refrigerated overnight, adjusted to pH 4·8–5·0 with 7·5N-NaOH, and 3 ml. were taken for enzymic treatment.

Asparagine. A sample of extract (2 ml.) was mixed with 2 ml. 4N-H_2SO_4 in a small tube fitted with a capillary condenser. The solution was heated in a boiling water bath for 3 hr., made up to 5 ml. and then 1 ml. portions were taken for ammonia estimation using 1 ml. alkaline metaborate, diffusion being allowed to proceed for 18–20 hr. at room temperature. Ammonia N in excess of the free ammonia + glutamine amide N was taken as asparagine amide N.

Alanine was estimated in extracts by means of ninhydrin oxidation and colorimetric estimation of the acetaldehyde produced (Folkes, 1953). The extract (0·5 ml.) was first dried *in vacuo* to remove ethanol. No correction was made for other acids, as chromatography showed that these were present in small amounts only.

Glutathione was estimated colorimetrically on 2 ml. extract by the nitroprusside reaction of Grunert & Phillips (1951), using a Unicam Absorptiometer with 1 cm. cells and an Ilford no. 624 (green) filter. Total glutathione was measured after reduction on a stirred mercury cathode for 10 min. at a current density of 3 mA/sq.cm. (Dohan & Woodward, 1939).

Chromatography of amino acids. Qualitative investigation of the amino acids in the extracts was by partition chromatography on Whatman no. 1 paper using water-saturated phenol, containing 1% (v/v) acetic acid, as the ascending solvent phase. Extracts were de-acidified first with 1% $BaCO_3$ and papers were run for 5–6 hr., then dried and amino acids detected by spraying with 0·2% ninhydrin in aqueous butanol. Standards containing glutamic acid, aspartic acid and alanine, or glutamine and asparagine, were run on each paper.

Total carbohydrate was determined colorimetrically by the anthrone method developed by Trevelyan & Harrison (1952). Where yeast suspensions from manometric experiments were tested, 1 ml. of suspension was pipetted into 2 ml. 4N-H_2SO_4 to kill the cells and then diluted to 25 ml. with water. In some cases 0·1 ml. suspension only was used with appropriately smaller amounts of acid and water. Yeast residues were diluted, 0·5–50 ml., before analysis. The volume of diluted carbohydrate taken for the reaction was always 1 ml. and the colour intensity was measured in a Unicam Absorptiometer using 1 cm. cells and an Ilford no. 607 (orange) filter. Calibration was against glucose, and all results are expressed on this basis. Mannose was found to give much less intense colour than glucose (approx. 57%, cf. Trevelyan & Harrison, 1952, who found 55%) and correction was made for this when comparing estimates of insoluble carbohydrates by the anthrone and copper reagents.

Soluble sugars. The micro modification by Willis & Yemm (1954) of the method of Somogyi (1945) was used for the estimation of soluble sugars by measurement of the reducing power of the extracts both before and after hydrolysis. 'Glucose' was estimated by direct measurement of reducing power of 1 ml. portions of an extract previously neutralized and diluted 2·5 times. 'Sucrose' was determined similarly, but the acid extracts were heated for 10 min. at 100° before neutralizing and diluting; a correction for reducing power due to 'glucose' was of course made. Trehalose was estimated from the reducing power of extracts hydrolysed with 2N-H_2SO_4 at 100° for 3 hr.; 3 ml. of the diluted and hydrolysed extract remaining from the estimation of asparagine were neutralized and made to 3·5 ml., 1 ml. portions being taken for the Somogyi estimation.

Insoluble carbohydrates were fractionated by the methods of Trevelyan & Harrison (1952). The glycogen + mannan fraction, extracted from 5 ml. residue with KOH, and then H_2SO_4, and the mannan fraction, precipitated with Fehling's solution from a KOH extract of 5 ml. residue, were each hydrolysed for 2 hr. at 100° in the presence of 2N-H_2SO_4, then neutralized and diluted to 375 ml. Portions (1 ml.) were then taken for estimations of reducing power as above.

Chromatographic investigation of carbohydrate fractions. From about 4·5 g. fresh weight of sugar-fed yeast, three fractions were separated by the methods described above. These fractions were soluble sugars, glycogen + mannan, and mannan. The polysaccharide fractions were hydrolysed for 3 hr. at 100° with 2N-H_2SO_4 and neutralized with $BaCO_3$. All were investigated by descending partition chromatography on Whatman no. 1 paper using butanol:pyridine:water (10:3:3, v/v) or ethyl acetate:acetic acid:water (9:2:2, v/v) as solvents and 1% *p*-anisidine hydrochloride in aqueous butanol for detection.

Calculation of results. Both fresh weight and dry weight are likely to change during experimental treatment of the yeast and so do not provide reliable bases for the calculation of results. All results have therefore been calculated on the basis of 100 ml. yeast suspension before it was diluted by the addition of phosphate solutions. The suspension generally contained 2 g. fresh weight of yeast/100 ml., but an exception was in the sugar-feeding experiment (see Table 2) where the suspension was approximately 1% before feeding and increased during the experiment.

RESULTS

The effect of ammonium ions on the respiration of nitrogen-deficient yeast

Numerous experiments have shown that the addition of an ammonium salt, such as ammonium phosphate, to nitrogen-deficient yeast results in a

considerable and very rapid rise in its rate of cellular respiration. Some typical measurements of oxygen uptake are given in Fig. 1.

The respiratory rate of nitrogen-deficient yeast, freshly transferred from a sugar-rich medium, shows at first a gradual decline, but after several hours it becomes almost constant. The addition of sodium phosphate to such a yeast causes no significant change in respiratory rate; this is apparent from the small scatter of values for both untreated and sodium phosphate treated yeasts in Fig. 1. The addition of ammonium phosphate, on the other hand, results in a sharp rise in the rate of oxygen uptake to 3 or 4 times its previous value. This high rate is maintained for about half an hour and then falls, rapidly at first and subsequently more slowly, to a value about double that of the yeast without added ammonium. This steady, or comparatively steady, rate of respiration is reached about 3 hr. after the addition of the ammonium solution. It is apparent from the curves given in Fig. 1 that the effect upon respiration of adding ammonium ions is extremely reproducible and relatively independent of the time of this addition.

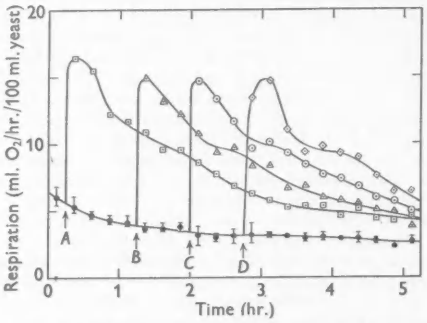

Fig. 1. The effect of ammonium ions on the rate of oxygen uptake of nitrogen-deficient yeast. ● Mean respiration rates, yeast untreated or with added sodium phosphate (range of values indicated). ⊡, △, ⊙, ◇ Respiration of individual samples treated at *A*, *B*, *C* and *D* respectively with a mixture of sodium and ammonium phosphates equivalent to 14 mg. N/100 ml. yeast suspension.

Data collected with *Torulopsis utilis* and other yeasts are summarized in Table 1, in which mean rates of respiration are given for a number of experiments. It can be seen that ammonium ions have a similar effect upon the respiration of all three yeasts tested, although some differences in the extent of response are apparent between baker's yeast and the two varieties of *Torulopsis utilis*. *T. utilis* B.P.60 was used for all the other experiments described in this paper.

The high degree of reproducibility in the experimental results is apparent from the data for *T. utilis* given in Table 1. The variation from experiment to experiment is small, and much of it is probably due to differences in the time of addition of ammonium phosphate. The two sets of results given for Expt. 9 illustrate this; the second series (*b*) was given by a sample treated with ammonia nitrogen 2 hr. after the first (*a*), and the differences between these two are nearly as large as any between pairs of experiments. Much of the variation in this and other experiments (see also Fig. 1) can be attributed to the differences between the rates of the nitrogen-deficient controls. These differences,

Table 1. *Respiration and the synthesis of glutamic acid in food yeasts and in baker's yeast*

In each experiment 3 ml. of 2% yeast suspension were treated at 25° in Warburg flasks with 0·5 ml. 0·06 M-Na_2HPO_4 (−N) or with 0·5 ml. of a mixture of equal parts of 0·06 M-$(NH_4)_2HPO_4$ and 0·06 M-Na_2HPO_4 (+N). O_2 uptake was measured for 2 hr. and then glutamic acid, free and as glutamine, was determined by means of glutamic decarboxylase.

Yeast	Expt. no.	Respiration (ml. O_2/hr./100 ml. suspn.) 1st hr. +N	1st hr. −N	2nd hr. +N	2nd hr. −N	Glutamic acid (mg. N/100 ml. suspn.) after 2 hr. +N	Glutamic acid (mg. N/100 ml. suspn.) after 2 hr. −N
Torulopsis utilis (BP60)	1	12·4	4·4	9·1	3·3	1·35	<0·02
	2	13·9	4·4	9·9	3·4	—	—
	3	14·1	4·9	9·7	3·5	1·44	0·02
	4	12·6	4·3	9·1	3·1	—	—
	5	13·3	5·0	9·3	3·5	—	—
	6	13·7	6·1	9·8	3·4	—	—
	7	14·0	4·6	9·4	3·4	—	—
	8	13·3	4·3	9·2	3·4	1·20	<0·02
	9*a*	13·9	5·1	9·6	4·0	—	—
	9*b*	12·5	3·3	8·8	2·9	—	—
	10*a*	12·6	4·2	9·6	3·1	1·28	<0·02
T. utilis var. *major* (N.C.T.C. 6593)	10*b*	11·2	3·9	8·3	3·1	1·23	<0·02
Baker's yeast	11	19·5	8·0	13·5	6·8	0·85	<0·02

although small, appear to influence in a direct additive manner the rate observed after nitrogen has been supplied.

The variation between replicate samples of the same experiment is small; in a typical experiment (Expt. 1) nine samples of untreated yeast showed a coefficient of variation in respiratory rate of $\pm 1{\cdot}4\,\%$, and eight samples after addition of ammonium ions gave a coefficient of variation of $\pm 0{\cdot}8\,\%$. This very small variation made extensive replication unnecessary and indicates that the results of different experiments can be compared with some confidence.

The high rate of oxygen uptake in ammonium-treated yeast is accompanied by a marked increase in the rate of carbon dioxide production, but the results shown in Fig. 2 indicate a change in R.Q. as compared with the control. At first the R.Q. of the control is about 1·05 and thereafter falls gradually to a value of about 0·90 after 3 hr. Treatment with ammonium ions leads to a sharp fall to about 0·85 and a subsequent slight rise to a value approaching that of the control.

The relation between the increase of respiration and the uptake of ammonium ions

When replicate samples of a nitrogen-deficient yeast suspension are treated with solutions containing various amounts of ammonium ions a close relationship is found between the increase of respiration and the amount of ammonium ions taken up by the yeast. The results of two similar experiments, shown together in Fig. 3, demonstrate this relationship. The rate of oxygen uptake after ammonium salts have been added is shown in detail for four levels of ammonia nitrogen in Fig. 3*a*, and in Fig. 3*b* total oxygen uptake over 3 hr., is plotted against the level of ammonium ion added to the yeast. From this it appears that there is a critical level of ammonium (approx. 10 mg. N/100 ml. under these conditions) below which variation in ammonium has a marked effect on respiration, and above which further increases of ammonium have no appreciable effect. Consideration

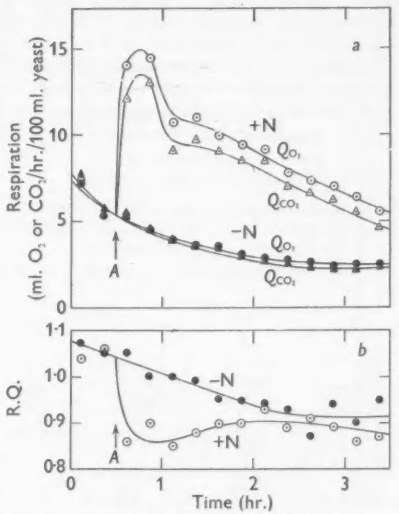

Fig. 2. Changes in respiratory activity of nitrogen-deficient yeast and of yeast treated at *A* with ammonium phosphate (14 mg. N/100 ml. yeast). *a*: rates of oxygen uptake and carbon dioxide output (each based on duplicate samples). *b*: changes in R.Q. (calculated from data of *a* above).

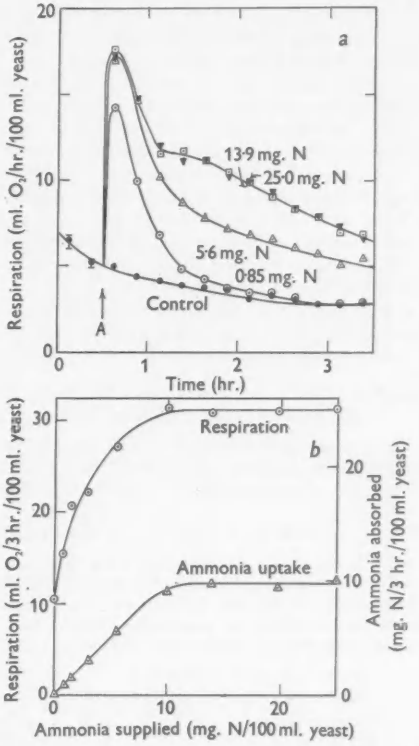

Fig. 3. The effect of varying level of ammonium N upon the respiration and ammonia uptake of nitrogen-deficient yeast. *a*: changes in respiration rate after adding (at *A*) mixtures of sodium and ammonium phosphates containing the stated amounts of N/100 ml. yeast suspension. The control was treated with sodium phosphate only. *b*: uptake of oxygen and ammonia N in 3 hr. after treatment with various levels of ammonium phosphate. The respiratory results are derived in part from those given in *a* (above). Ammonia uptake was estimated from the free ammonia remaining after 3 hr.

of the data on ammonium taken up by the yeast, also presented in Fig. 3*b*, shows that this critical level coincides with the level of ammonium which can be completely assimilated by the yeast during the period of the experiment. Below the critical level, all the ammonium is absorbed, while an increase in concentration above the level results in no further uptake of ammonium. The increase in respiration can therefore be related to the absorption of ammonium ions.

The curves of Fig. 3*a*, showing oxygen uptake at different levels of ammonium supply, support this interpretation. Little difference is apparent in the curves for 13·9 and 25·0 mg. N/100 ml. during the 3 hr. period, and in this range of concentration free ammonia is still available at the end of the experiment. At 5·6 mg. N/100 ml., the ammonium supply is adequate to maintain a high rate of oxygen uptake over the first half hour only, and the respiration rate subsequently falls to a lower level than that observed at 13·9 or 25·0 mg. N/100 ml. In this case all the ammonia nitrogen was exhausted by the end of the experiment. At the lowest ammonium level the highest rates of respiration are never reached, or at least not maintained over 15 min., and there is every indication that the ammonia nitrogen is rapidly utilized; the rate of oxygen uptake falls to that of the control after about 1·5 hr.

A level of about 14 mg. N/100 ml. yeast has been used in all experiments in which an adequate ammonium supply was required.

The effect of feeding with sugar upon the composition and metabolism of Torulopsis utilis

The physiological state of the yeast has a decisive effect on the metabolic changes which follow the addition of ammonia. When yeast is transferred from the complete nutrient solution *A* to one containing nutrient salts only (solution *C*), its respiration is not significantly affected by the presence or absence of ammonium salts. If, however, a brief treatment in the nitrogen-deficient, high-carbohydrate solution *B* is interposed, a typical increase in the rate of oxygen uptake is observed as already described. The changes which take place during the period of sugar feeding in solution *B* have been investigated by removing samples at 4-hourly intervals for analysis and measurement of +N and −N respiration. The results of one such experiment are given in Table 2. A small correction for sampling errors has been made by assuming that the total nitrogen of the yeast remained constant throughout the period of sugar feeding.

An important effect of sugar feeding upon the composition of the yeast is upon the partition of nitrogen between soluble and insoluble (mainly protein) fractions. During the first 4 hr. the level of soluble nitrogen falls to about half its original value and thereafter is maintained at this low value. A further result of sugar feeding is an increase both in dry weight and in carbohydrate content. The increase of dry weight cannot be attributed entirely to the accumulation of carbohydrates, and, while it may be partly due to the uptake of mineral ions, it is probable that formation of fat reserves also occurs. Some further evidence on this point will be given later, but it is well established that in some yeasts fat may constitute up to 12% of the fresh weight, and the amount is greatest under conditions of high carbohydrate, high phosphate, low nitrogen and high aeration (Smedley-Maclean & Hoffert, 1923).

Successive periods of sugar feeding lead to increased rates of oxygen uptake upon removal of the yeast to a sugar-deficient medium either with or

Table 2. *The effect of feeding with sugar on the composition and metabolism of yeast*

A 1% suspension of freshly grown *T. utilis* in soln. *B* was treated in the standard manner for the preparation of N-deficient yeast (see Material and Methods). At intervals, 25 ml. samples were taken, washed at the centrifuge and re-suspended in 25 ml. soln. *C*. Portions of this suspension were used for analyses of composition, and for metabolic studies with or without ammonia as described in Table 1. O_2 uptake was measured for 3 hr. and ammonia assimilated was estimated from the residual ammonia at the end of this period.

Duration of sugar feeding (hr.) ...	*0·3*	*4·3*	*8·3*	*12·3*	*16·3*
Composition (mg./100 ml. yeast suspn.)					
Fresh weight	1210	1550	1920	2130	2320
Dry weight	201	300	379	463	520
Carbohydrate (anthrone method, as glucose)	37	107	170	207	239
Dry matter other than carbohydrate (by difference)	164	193	209	256	281
Soluble nitrogen	2·1	1·1	1·0	1·0	1·0
Protein nitrogen	11·1	12·1	12·2	12·2	12·2
Metabolism (in 3 hr./100 ml. yeast suspn.)					
Respiration, −N (ml. O_2)	4·8	10·5	11·4	12·8	13·4
Respiration, +N (ml. O_2)	4·9	14·6	24·8	30·3	34·8
Difference in respiration between +N and −N (ml. O_2)	0·1	4·1	13·4	17·5	21·4
Ammonia assimilated, +N (mg. N)	0·1	3·2	7·3	9·0	10·3

without ammonia nitrogen. While the growth of the yeast probably accounts largely for the increased respiration in the absence of ammonium, the increase of respiration of the ammonia-treated yeast is much greater, and large differences in respiratory rate between the two treatments soon develop. These differences are closely correlated with an increase in the amount of ammonia nitrogen assimilated by the yeast. Thus the yeast, soon after removal from the nitrogen-rich solution *A*, shows little difference in rate of respiration between the +N and −N treatments and only negligible amounts of ammonia nitrogen are taken up, while after further sugar feeding both the difference in rates of oxygen uptake and the amount of ammonium assimilated show large increases. It is noteworthy that the greatest of these increases coincides with a period (0–8 hr.) in which changes in carbohydrate, total dry matter and nitrogenous constituents are greatest. The results clearly indicate that a high reserve of carbohydrate together with a low level of soluble nitrogen are two important factors influencing the assimilation of nitrogen and the associated changes in cellular respiration.

Changes in the composition of Torulopis utilis *during the assimilation of ammonium salts*

The changes of some of the nitrogenous constituents of this yeast during active assimilation of ammonia, under conditions similar to those used here, have already been described by Roine (1947) and by Virtanen *et al.* (1949). In the experiments described in this section the analyses were extended to include the soluble and insoluble carbohydrates of the yeast in order to give a more comprehensive account of the metabolic changes and their relation to cellular respiration.

Several experiments have given essentially similar results and those from one experiment are presented in Figs. 4 and 5. Samples were taken for analysis before ammonia treatment and 0·5, 1, 2 and 3 hr. after the addition of ammonium phosphate. A yeast deprived of nitrogen was also sampled after 3 hr. The respiratory behaviour determined from parallel samples, has been summarized in part in Table 1 (Expt. 3) and was very similar to that illustrated in Fig. 2.

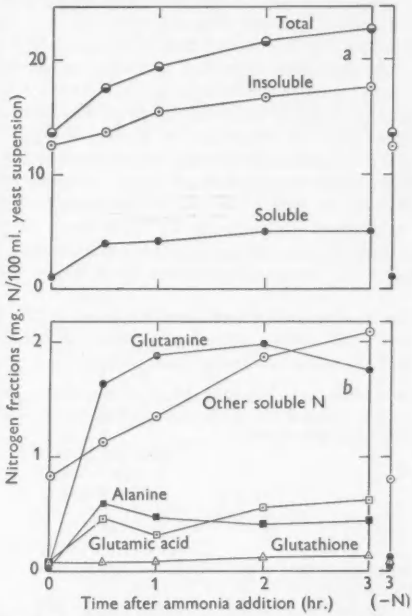

Fig. 4. Changes in nitrogenous fractions during the assimilation of ammonia N by nitrogen-deficient yeast. *a*: changes in total-nitrogen, insoluble- (protein) and soluble-nitrogen fractions. *b*: the major components of the soluble fraction. The corresponding levels in yeast treated with sodium phosphate (−N) for 3 hr. are indicated to the right of the figure.

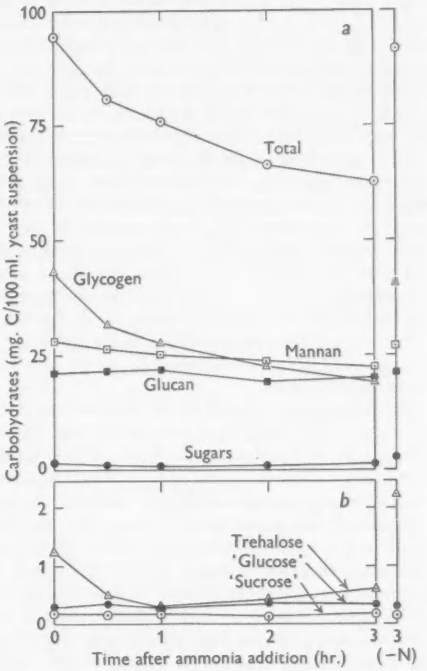

Fig. 5. Changes in carbohydrate fractions accompanying the changes in nitrogenous fractions shown in Fig. 4. *a*: the major carbohydrate fractions. *b*: the soluble sugars.

During the treatment with ammonium phosphate, nitrogen is assimilated by the yeast into a number of nitrogenous compounds. This is clear from Fig. 4. Both the insoluble and soluble nitrogen fractions show increases, although most of the increase in total nitrogen in the first half hour is attributable to the soluble fraction, whereas subsequently the insoluble fraction shows the main increase. As the soluble nitrogen has been corrected for free ammonia nitrogen, the increases in both total nitrogen and soluble nitrogen demonstrated here represent the incorporation of nitrogen into organic compounds and not merely the accumulation of ammonium ions within the cell. Indeed, as Roine (1947) has already reported, much of the increase in soluble nitrogen over the first 30 min. of assimilation is due to the synthesis of three compounds: glutamic acid, glutamine and alanine. In three similar experiments these compounds account for 90–100 % of the increase in soluble nitrogen and 65–70 % of the increase in total nitrogen over this first period, a period in which the rate of respiration is greatest. In later periods the levels of these compounds are, in general, maintained and further nitrogen assimilation is reflected mainly in increases in the insoluble protein nitrogen. This is especially marked in the period 0·5–1 hr. after addition of ammonia. Glutathione shows little change over the 3 hr. period of the experiment, and other nitrogenous compounds increase steadily throughout. The nature of these compounds was investigated both by examination for stable amide nitrogen and by chromatography. No amide other than glutamine could be detected in the former test so the traces of substances (R_F 0·48) appearing on the chromatogram may well have been glycine (R_F 0·49) and not asparagine (R_F 0·46). The only other coloured spot not accounted for by the analyses was one with R_F 0·70 (probably valine or methionine). In the −N yeast no appreciable change in any of the nitrogenous fractions was detectable over the period of the experiment.

The changes in the level of the carbohydrate fractions, given in Fig. 5, show that the assimilation of nitrogen is associated with high rates of carbohydrate breakdown. Glycogen is the main component disappearing, although mannan is also important. The composition of these two fractions was checked by chromatography of the hydrolysed fractions, and, as reported for baker's yeast by Trevelyan & Harrison (1952), they were found to yield predominantly (at least 70 %) glucose and mannose respectively. The experimental error in the determination of glucan renders accurate assessment impossible, but it seems unlikely that it can make more than a minor contribution to the total carbohydrate disappearance, and the fact that this fraction is localized in the outer cell wall (Northcote & Horne, 1952) also suggests that it is not readily mobilized. Changes in the soluble sugars are very small and only trehalose is of importance, and that mainly during the first 30 min. period. Only trehalose, together with small amounts of glucose, could be detected chromatographically and the identity of the fraction reported as 'sucrose' is uncertain. More striking changes in the soluble sugars are apparent in the −N yeast where trehalose accumulates while the insoluble carbohydrates show only small decreases.

An interesting feature of these results is that, while the +N yeast exhibits a rate of respiration approximately three times that shown by the −N yeast, the losses in total carbohydrate over 3 hr. are 10 times as great in the +N yeast as in the −N. A balance sheet of the carbon changes drawn up in Table 3 throws some further light on this problem. In the absence of a full identification of the nitrogenous and other constituents of the yeast, such a balance sheet can only be approximate. Of the soluble fraction only the 'other soluble nitrogen' was in doubt and this was assumed to have a C/N ratio of 4/1. The insoluble fraction was considered to be a typical plant cytoplasmic protein (cf. Lindan & Work, 1951; Yemm & Folkes, 1953) and its carbon content was calculated on this basis. It is evident that the synthesis of amino acids and of proteins represents an important drain upon the total carbon available; roughly twice as much is used for this purpose as is lost as respiratory carbon dioxide. In the −N yeast slight protein breakdown occurred and this has been allowed for in the balance sheet.

The carbon derived from losses in carbohydrates is insufficient to account for that used in the synthesis of the nitrogenous compounds and for respiration. Carbon from some other source must

Table 3. *Carbon balance sheet for yeast treated for 3 hr. with sodium phosphate (−N) or with a mixture of sodium and ammonium phosphates (+N)*

Calculations are made from the experimental data given, in part, in Table 1 (Expt. 3), and in Figs. 4 and 5. For details see Results section.

	Changes during 3 hr. (mg. C/100 ml. yeast suspension)			
	−N		+N	
Products				
Respiratory CO_2	5·9		14·6	
Syntheses				
Soluble amino acids	0·1	−0·5	11·3	26·7
Proteins	−0·6		15·4	
Total	5·4		41·3	
Losses				
Carbohydrates	3·0		30·4	
Other C compounds (to balance)	2·4		10·9	
Total	5·4		41·3	

also be available for metabolism and, as already suggested, fats may be important in this connexion. No direct determinations of fat content were made in these experiments, but an indirect estimate may be made from the R.Q. measurements after correcting for the changes in degree of oxidation involved in the synthesis of amino acids and proteins. Assuming an R.Q. of 0·7 for complete fat oxidation in the yeast (Daubney & Smedley-Maclean, 1927) it was calculated that over the 3 hr. period of the experiment, fat contributed about 0·4 mg. carbon in the −N yeast and 5·9 mg. carbon in the +N yeast. These estimates are lower than the estimates of 'other carbon compounds' made from the balance sheet: but it seems probable that fat contributes to the respiratory substrate under these conditions.

From the above it is apparent that the high rates of oxygen uptake accompanying nitrogen assimilation in low nitrogen yeasts are associated with the utilization of fats and carbohydrates, chiefly glycogen and mannan, and with the synthesis of soluble amino acids and, later, of proteins. Of the soluble compounds glutamic acid and glutamine are important and it is of interest that the accumulation of these compounds can be shown to occur in ammonium-treated samples not only of two strains of *Torulopsis utilis* but also of baker's yeast (see Table 1).

The uncoupling of nitrogen assimilation and respiration by means of 2:4-dinitrophenol

From these results it is evident that the assimilation of ammonia nitrogen, involving the synthesis of amino acids and proteins, is closely coupled with cellular respiration. Clifton (1946) has shown that several compounds, such as 2:4-dinitrophenol (DNP), strongly inhibit assimilation in micro-organisms under conditions in which respiration is much less affected. The observations of Massart & Heymans (1950*b*) with baker's yeast suggest a similar effect of dinitrophenols on the assimilation of nitrogen. After the work of Loomis & Lipmann (1948), it has been generally supposed that the differentiating effect of DNP on assimilation and respiration arises from an uncoupling of oxidation and phosphorylation, since the latter is now regarded as one of the chief ways in which respiratory energy is transferred to anabolic processes of the cell. It was of interest, therefore, to examine the effects of DNP on the metabolism of food yeast under some of the different conditions of nutrition which have been studied here.

For this purpose manometric experiments were performed in which various levels of DNP were added together with the sodium phosphate or sodium and ammonium phosphate mixtures. Measurements of oxygen uptake were continued for 3 hr. after the addition, and samples were then removed from the manometric flasks for estimates of carbohydrate loss, ammonia nitrogen assimilated, and of glutamic acid, free and as glutamine. Curves of oxygen uptake at three levels of DNP are given in Fig. 6 and the combined results of three similar experiments are shown together in Fig. 7.

It is apparent from the graphs in Fig. 6 that the addition of DNP to nitrogen-deficient yeast in the absence of added ammonium salts leads to very marked increases in respiration, of the same order as

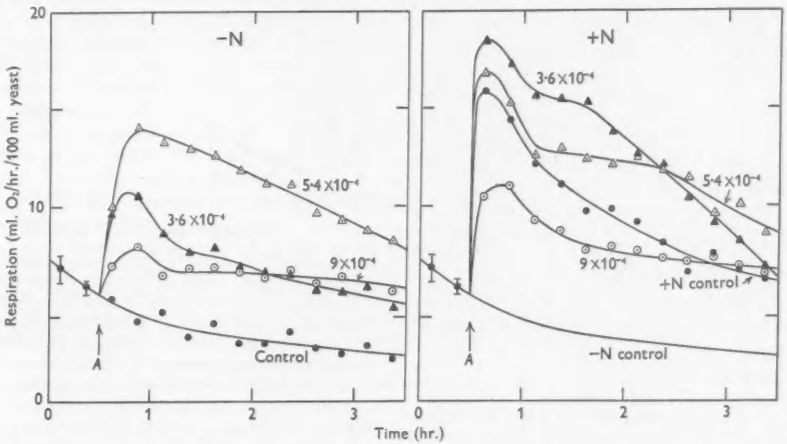

Fig. 6. Changes in respiration rate after adding (at *A*) DNP at the final molar concentrations shown, together with sodium phosphate (−N) or a mixture of sodium and ammonium phosphates (+N) to nitrogen-deficient yeast. The controls had no DNP addition.

those observed after addition of ammonia nitrogen in the absence of DNP, but somewhat different in form. These increases in the rate of oxygen uptake are greatest at the $5{\cdot}4 \times 10^{-4}$M level of DNP, and higher concentrations give progressively smaller increases: an inhibitory effect on oxygen uptake has been observed in other experiments at $1{\cdot}5 \times 10^{-3}$M and above.

When the data in Fig. 6 for samples in which DNP and ammonium were added together are considered, it is apparent that the separate effects of DNP and ammonia are not additive when the two are supplied together. Moreover, higher rates of oxygen uptake are observed with $3{\cdot}6 \times 10^{-4}$M DNP than with $5{\cdot}4 \times 10^{-4}$M, at least for the first 2 hr. after addition of DNP, the latter concentration giving somewhat higher rates subsequently. At 9×10^{-4}M the DNP is definitely inhibitory as compared with the +N control, although it still allows slightly higher rates of respiration than the same level of DNP gives in the −N series. The relationships underlying these data are obviously complex, but the chief features are more apparent from Fig. 7, where total changes over the 3 hr. after DNP addition are summarized.

The difference in sensitivity to DNP of the respiration of +N- and −N-treated yeasts can be seen from Fig. 7*a*. The +N yeast has a maximum oxygen uptake at $1\text{–}2 \times 10^{-4}$M DNP and a gradually decreasing rate at higher levels, whereas the optimum DNP level for the −N yeast is at about 5×10^{-4}M. On the other hand, far larger increases in respiration are observed in the −N yeast, although it never reaches the high levels of oxygen uptake observed in the +N series. The subsequent decline in rate of respiration in both +N and −N series is similar to that observed by Simon (1953*a*, *b*) for yeast with 3:5-dinitro-*o*-cresol in the presence and absence of external carbohydrate and suggested by him to be due to inhibition of electron-transfer systems in the yeast. The slight divergence in the two curves given in Fig. 7*a* is not necessarily in conflict with this hypothesis, as it may be due to slight differences in pH between the +N and −N cultures, leading to differences in the effective concentrations of DNP in the two series (Simon & Beevers, 1952). While all samples started at pH 6·0 and the pH of the −N series remained unchanged throughout the experiment, that of the +N series tended to become more acid in spite of the presence of phosphate buffer and pH's as low as 5·6 were recorded at the end of the experiment.

Definite evidence is given in Fig. 7*b* for an uncoupling of nitrogen assimilation and respiration. Low levels of DNP, while leading to somewhat increased rates of respiration, cause marked decreases in the rates of assimilation of nitrogen as evidenced both by total uptake of ammonia nitrogen and by the synthesis of glutamic acid. Nitrogen assimilation is almost completely inhibited at 10^{-3}M DNP, while at this level oxygen uptake is still 70% of that in the +N control.

The data for losses of carbohydrate during the 3 hr. of DNP treatment and presented in Fig. 7*c* are of interest in comparison with those of oxygen uptake given in Fig. 7*a*. Over the range of lower levels of DNP the effects are essentially similar, but at higher levels they differ in that, whereas the curve of oxygen uptake shows a pronounced maximum at 5×10^{-4}M DNP, the curve of carbohydrate loss continues to climb at this point and tends towards the steady level achieved at about the same DNP concentration by the +N series. Thus over a wide range of the higher DNP concentrations, carbo-

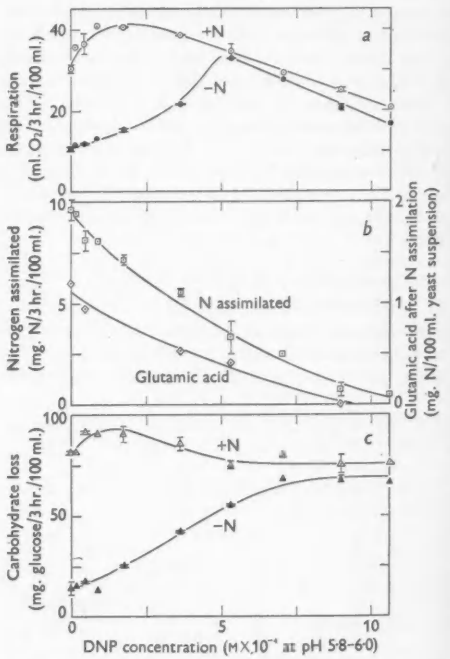

Fig. 7. The effect of DNP upon the metabolism of nitrogen-deficient yeast treated at the same time with sodium phosphate (−N) or with a mixture of sodium and ammonium phosphates (+N). All results represent total changes in 3 hr. after the addition of DNP and are from three similar experiments. Where results at any concentration of DNP are derived from more than one experiment the range of values is shown. *a*: total oxygen uptake/3 hr. in +N and −N yeast. These results are derived in part from those of Fig. 6. *b*: total ammonia N assimilated and increase of glutamic acid N in 3 hr. in the +N yeast. *c*: losses of total carbohydrate in +N and −N yeasts in 3 hr.

hydrate losses are being increased or maintained in both −N and +N conditions while oxygen uptake steadily decreases with increasing DNP. This implies an increasing component of fermentation; the ability of DNP to promote fermentation has been noted for yeast by Clifton (1946) and Simon (1953*a*, *b*) and for higher plants by Beevers (1953).

It is clear, therefore, that the process of assimilation of nitrogen responds in a similar manner to other assimilation processes (Clifton, 1946) to the action of DNP. The most important similarity is that an uncoupling of respiration and assimilation is apparent, both in the sense that assimilation is inhibited while oxygen uptake is little affected, and also that the high rates of respiration normally associated with the assimilation of ammonia nitrogen can be induced by DNP in the absence of ammonium.

DISCUSSION

The experiments described here have shown that, during the assimilation of ammonia nitrogen, there is at first a synthesis of amino acids and amides, largely identified as glutamic acid, glutamine and alanine. To this extent the results are in agreement with those of Roine (1947), who used somewhat different methods, and the three constituents given above account for 65–70% of the total nitrogen assimilated over the initial period of 30 min. Subsequently, the synthesis of proteins predominates, although the soluble nitrogenous fractions are maintained at a high level. Associated with these syntheses, high rates of cellular respiration are always observed, together with a breakdown of carbohydrates, mainly glycogen and mannan, in the yeast. There is some evidence that fats are also actively metabolized during the period of rapid assimilation of nitrogen. Many of the experiments indicate that these changes are coupled in the cellular mechanisms; for example, the increase in oxygen uptake can be closely correlated with the amount of nitrogen assimilated, which itself depends upon the level of available carbohydrate reserves. Moreover, uncoupling agents, such as DNP, are found to inhibit nitrogen assimilation while promoting oxygen uptake.

The important position of glutamic acid is apparent from the experimental results in which glutamine, together with free glutamic acid, are at first the chief products of the assimilation of nitrogen. Enzymic systems which catalyse the primary synthesis of both the free amino acid and the amide from ammonia nitrogen, have been separated from yeast by Adler, Günther & Everett (1938) and by Elliot (1951). In addition, it is well established that glutamic acid reacts in the synthesis of many amino acids by transamination and other reactions (Braunstein, 1947; Tatum, 1949). Transaminases leading to the synthesis of alanine and aspartic acid have been demonstrated in yeast by Roine (1947), and it is probable that alanine detected in the present experiments was formed in this way. Extensive synthesis of other amino acids is clearly indicated by the increase in protein in the later stages of assimilation, and recent theories (Fruton, 1950; Hanes, Hird & Isherwood, 1950) suggest that glutamine may be an important reactant in the formation of peptide bonds during this synthesis of protein. It is noteworthy that, despite the drain upon them by these reactions, both glutamic acid and glutamine are maintained at a high level within the cell throughout the period of active protein synthesis.

The highest rates of respiration are observed during the initial period of synthesis of amino acids and amides, and the relationship between this synthesis and the breakdown of carbohydrates is of primary importance. Elliot (1951) has shown that the synthesis of glutamine from glutamic acid and ammonia is enzymically coupled with the breakdown of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). Since, during glycolysis and respiration (the term 'glycolysis' is used throughout this discussion in a general sense to denote breakdown of sugars to C_3 compounds common to both fermentation and respiration), ATP is regenerated from ADP (Lipmann, 1941), this may constitute a direct energy link between cellular oxidations and the synthesis of the amide, and hence, by transfer reactions, of peptide bonds. The importance of phosphorylations as a link between respiration and assimilation, or other synthetic reactions, is widely accepted and has been discussed in relation to peptide synthesis by Lipmann (1949). In the present experiments further evidence of the linkage was obtained by the use of DNP. Although the exact mode of action of this inhibitor is uncertain, it is well established that it uncouples oxidation and phosphorylations in cell-free preparations. The selective inhibition of ammonia uptake by low concentrations of DNP is fully consistent with the importance of phosphorylations in the assimilation of nitrogen.

There are, however, two other ways in which the assimilation of nitrogen may interact with glycolysis and respiration. The synthesis of glutamic acid by the action of glutamic acid dehydrogenase is a reductive amination involving the oxidation of a phosphopyridine nucleotide. It is thus directly linked through the hydrogen-carrier systems with the oxidation of substrates in the cell. Hence the system synthesizing glutamic acid can be regarded as an accessory hydrogen-acceptor diverting electrons at a high level of oxidation–reduction energy and thus excluding oxidative phosphorylations normally associated with aerobic systems. As

the amino groups of many amino acids may be derived from glutamic acid, these reactions may achieve significant proportions during protein synthesis. For example, it can be calculated from the analytical data presented in Fig. 4 and Table 1, that, during a period of nitrogen assimilation lasting 3 hr., approximately one-sixth of the total electron transfer is utilized for amino acid synthesis rather than passed to atmospheric oxygen.

A third way in which the assimilation of nitrogen and the metabolism of carbohydrates are closely related is in the carbon skeletons of newly synthesized amino acids. Glutamic acid and alanine, the two amino acids most rapidly synthesized in the early stages of assimilation, are in all probability derived from α-oxoglutaric acid and pyruvic acid, both of which are well established intermediaries in the respiratory breakdown of carbohydrates. Krebs, Gurin & Eggleston (1952) have recently discussed the occurrence and significance of the tricarboxylic acid cycle in yeast and have emphasized its importance as a source of reactants for cellular syntheses. The present results strongly support this view; the carbon balance sheet drawn up in Table 3 shows that, of the increases in carbon consumption during nitrogen assimilation, 2–3 times as much is utilized for amino acid and protein syntheses as undergoes complete oxidation to carbon dioxide. A high proportion of the carbon diverted from oxidation in this way is derived from acids of the tricarboxylic acid cycle; for example, 26% can be accounted for by the glutamic acid and alanine accumulated in the soluble fraction alone.

The data presented here offer many similarities to those now available for higher plants and for other micro-organisms. Willis (1951) has shown with roots of barley that high rates of respiration and of carbohydrate breakdown are associated with the assimilation of ammonium and nitrate nitrogen, and that a primary synthesis of glutamine, together with smaller amounts of asparagine, occurs. Syrett (1953*a*, *b*) has obtained very similar results with *Chlorella vulgaris*. Although in the present experiments with yeast no appreciable amounts of asparagine were found, Virtanen *et al.* (1949) have given some evidence that this amide is formed in *Torulopsis utilis* when the latter is supplied with nitrate. Marked increases in oxygen uptake under some conditions have been observed on treatment with ammonia of other yeasts (Winzler *et al.* 1944; Runnström, Brandt & Marcuse, 1940; Massart & Heymans, 1950*a*) and some bacteria (Armstrong & Fisher, 1947; McLean & Fisher, 1947; Burris & Wilson, 1952). Increases of anaerobic glycolysis under similar conditions have also been recorded in yeast by Smythe (1939) and Winzler *et al.* (1944).

These observations of increased rates of carbohydrate breakdown and of oxygen uptake during the assimilation of nitrogen are relevant to the general problem of the regulation of cellular respiration. Much recent evidence converges to show that the rate of cell respiration is closely dependent on assimilation and other endergonic reactions. For example, high rates of respiration or glycolysis have been found to accompany not only nitrogen assimilation but also the assimilation of glucose in yeast (Simon, 1953*a*), the anabolic processes of young seedlings (Folkes, Willis & Yemm, 1952), the accumulation of salts by roots (Milthorpe & Robertson, 1948; Robertson, Wilkins & Weeks, 1951), and, in animals, muscular (Fenn, 1927) and nervous activity (McIlwain, 1950). Current theories (see, for example, Dixon, 1949) have laid great stress on phosphorylation as the underlying mechanism whereby catabolic processes are regulated to meet the energy requirements of synthesis and other functional activities of the cell. It is generally considered that the reversible reactions which make up the early stages of carbohydrate breakdown are controlled by the availability of free phosphate and/or suitable phosphate acceptors which may, in turn, depend upon their rates of formation from high energy phosphate compounds in endergonic reactions. These views have been put for nitrogen assimilation by Sussman & Spiegelman (1951) and by Syrett (1953*b*), and the increases in respiration and glycolysis observed in the present experiments with ammonia and with DNP can be interpreted along these lines: both DNP and ammonium may decrease the level of high-energy phosphate compounds, the one by uncoupling their formation and the other by providing phosphate-acceptor reactions in protein synthesis.

On such a theory, however, it is difficult to interpret completely the effects of DNP, both in the presence and absence of ammonia. There is some evidence (see Fig. 7) that both the uptake of oxygen and the breakdown of carbohydrate are inhibited by higher levels of DNP, although glycolysis is clearly less sensitive than respiration especially in the absence of ammonia. These inhibitory effects of DNP are particularly evident on the high metabolic rates observed during the assimilation of nitrogen and it is probably under these circumstances that other DNP-sensitive reactions become rate-limiting in carbohydrate breakdown and oxygen uptake. For example, Haas, Harrer & Hogness (1942) have shown that 10^{-3} M 2:4-dinitro-*o*-*cyclo*hexylphenol at pH 8·3 causes 70% inhibition of cytochrome reductase, and Simon (1953*c*) has suggested that this inhibition of flavoprotein enzymes, or of some other system, by dinitrophenols may be important during the high rates of respiration associated with glucose assimilation. A similar interpretation could apply to the present experiment. The action of DNP upon the glycolytic systems is more uncertain;

Bonner (1949) has suggested that in higher plants, a decrease in high-energy phosphate compounds may limit the initial phosphorylation of carbohydrates. But it is clear from our experiments that many other interactions are involved. The most substantial inhibition of carbohydrate breakdown by DNP occurs between $1{\cdot}5 \times 10^{-4}$M and 5×10^{-4}M in the presence of ammonia, and is associated with a marked decrease in the rate of nitrogen assimilation. Over the full range of DNP concentrations investigated in the presence of ammonia, there is a striking change in the fate of the products of glycolysis: in the absence of DNP, as already stated, about two-thirds of the glycolysis products are utilized for the synthesis of amino acids and proteins whereas, with 10^{-3}M DNP, only negligible amounts are utilized in this way, and at least two-thirds of the products of glycolysis appear to be diverted to fermentation reactions. While this implies an extensive switch in the fate of these products and in the enzymic mechanisms involved, there are several similarities between reductive amination and the dismutations associated with the formation of ethanol in fermentation. Both reactions involve a diversion of glycolysis products and of electrons from the normal aerobic channels of oxidative phosphorylation. In this regard the increased rates of glycolysis during the assimilation of nitrogen present a parallel with the Pasteur effect, in which low oxygen tension or inhibition of cellular oxidations leads to alcoholic fermentation and an increase in the rate of carbohydrate breakdown. However, in contrast to fermentation, the reductive synthesis of glutamic acid takes place under aerobic conditions and competes successfully with the electron-transfer systems leading to atmospheric oxygen. It seems probable that the occurrence of such powerful alternative electron-acceptor reactions will increase the proportion of oxidized to reduced phosphopyridine nucleotides and hence will promote the activity of triose phosphate dehydrogenase in glycolysis, whereas anaerobic fermentation will have an opposite effect. In addition, the assimilation of nitrogen generates high energy phosphate acceptors, leading to the synthesis of amide and peptide bonds. These multiple effects on glycolysis and cellular oxidations may well account for the observations that the rate of carbohydrate breakdown is higher during the assimilation of ammonia than it is in yeast treated with DNP alone, and that this rate decreases when nitrogen assimilation is itself inhibited by DNP.

There is little doubt that phosphorylation reactions constitute an important link between the breakdown of carbohydrate in respiration and the endergonic reactions of the cell. However, the present experiments strongly suggest that when the endergonic reactions involve extensive synthesis, other interactions may also be important in regulating cellular respiration and glycolysis. A large part of the products of glycolysis may be used for these syntheses and associated reduction reactions may alter the oxidation–reduction conditions within the cell. All of these components are closely integrated and are brought into action during nitrogen assimilation and protein synthesis. They may be largely responsible for the interdependence, commonly observed in higher plants and microorganisms, between respiration and the metabolism of nitrogen.

SUMMARY

1. Nitrogen-deficient cultures of food yeast, *Torulopsis utilis*, suspended under aerobic conditions in a carbohydrate-free medium, have been shown to respond rapidly to the addition of ammonium phosphate by increasing their rate of oxygen uptake to 2 or 3 times the previous level. Their carbon dioxide output is also increased but the R.Q. falls from 1·05 to about 0·85.

2. This increase in respiration is accompanied by the assimilation of ammonia and a close quantitative relationship between these metabolic processes can be demonstrated.

3. Nitrogen deficiency can be induced by culture of the yeast in a high-carbohydrate, nitrogen-free solution, and its onset is associated with a marked increase in the level of stored carbohydrate and other materials within the yeast, and with an increase in the protein content at the expense of the soluble nitrogen compounds. This gives a greater capacity for nitrogen assimilation upon treatment with ammonia.

4. The assimilated nitrogen appears mainly as glutamine, alanine, glutamic acid and, later, as protein. These syntheses are accompanied by the breakdown of stored carbohydrates, mainly glycogen and mannan, and of fats.

5. 2:4-Dinitrophenol (DNP) has been found to inhibit the assimilation of nitrogen and to cause increased oxygen uptake and carbohydrate breakdown. At higher concentrations of DNP oxygen uptake also is inhibited and fermentation reactions predominate.

6. These results are discussed with special reference to the problem of the regulation of respiration and glycolysis within the cell. It is suggested that the assimilation of nitrogen may increase the rate of carbohydrate breakdown and of respiration in three ways: the synthesis of amide and peptide bonds may lower the level of high-energy phosphate compounds, the diversion of electrons for reductive amination reactions may alter the oxidation–reduction conditions within the cell, and the synthesis of amino acids and proteins may make large demands upon the organic acids produced in the course of glycolysis and respiration.

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The Estimation of Carbohydrates in Plant Extracts by Anthrone

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The green colour produced when carbohydrates are heated with anthrone in acid solution was first used as a qualitative test by Dreywood (1946); since then anthrone has been extensively employed as a convenient and specific reagent for the colorimetric estimation of a variety of carbohydrates (see, for example, Viles & Silverman, 1949; Seifter, Dayton, Novic & Muntwyler, 1950; Trevelyan & Harrison, 1952). There has, however, been no systematic investigation of the reaction with the different hexose and pentose carbohydrates which may be encountered in biological material. Moreover, different conditions have been used for the reaction and the extent to which sugars may interfere with one another when present in mixtures is uncertain. Johanson (1953*a*) has, in fact, recently stated that

arabinose, although itself giving rise to relatively little colour under the conditions employed for the estimation of glucose, nevertheless interfered seriously in the estimation of a mixture of these two sugars. An investigation of the reaction has therefore been carried out with a range of naturally occurring sugars and sugar mixtures, with a view to assessing the reliability of the anthrone reagent for the analysis of carbohydrates found in plant tissues.

MATERIALS AND METHODS

Anthrone reagent. This was prepared as described by Trevelyan & Harrison (1952) by dissolving 0·2 g. of anthrone in 100 ml. of H_2SO_4, made by adding 500 ml. of conc. acid to 200 ml. of water. The reagent was allowed to stand for 30–40 min. with occasional shaking until it was perfectly clear. Recrystallization from benzene and light petroleum was necessary with some commercial samples of anthrone. The reagent was freshly prepared each day and used within 12 hr.

Sugars. The purest commercially available preparations of sugars (British Drug Houses Ltd., L. Light and Co. Ltd., Roche Products Ltd.) were used. Water contents were determined on samples of the sugars by drying to constant weight at 80° or, for rhamnose hydrate, for short periods at 105°.

Reaction conditions. The reaction was carried out under conditions similar to those used by Trevelyan & Harrison (1952). The anthrone reagent (5 ml.) was pipetted into thick-walled Pyrex tubes (150 × 25 mm.) and chilled in ice water. The solution under test (1 ml.) was layered on the acid, cooled for a further 5 min. and then thoroughly mixed while still immersed in ice water. The tubes were loosely fitted with corks, heated as required in a vigorously boiling, constant-level water bath and then cooled in water for 5 min.

Colorimetry. For routine purposes a photoelectric colorimeter (Evans Electroselenium Ltd.) was used, with $\frac{5}{8}$ in. diameter tubes and with an Ilford Spectrum Orange filter no. 607 (max. transmission at 600 mμ.). The measurements of test solutions and of reagent blanks were made against water as a reference. The relation between scale readings and amounts of sugars was not strictly linear, and it was necessary to use calibration curves for the different sugars. Absorption spectra were determined in a spectrophotometer (Unicam SP. 500) with a 1 cm. cell.

Paper chromatography of sugars. This was carried out by descending filter-paper chromatography according to the methods of Partridge (1948) and Hough, Jones & Wadham (1950). The solvents employed were *n*-butanol:pyridine:water (10:3:3, v/v), *n*-butanol:ethanol:water (40:11:19, v/v) and acetic acid:ethyl acetate:water (2:9:2, v/v). *p*-Anisidine hydrochloride (1%, w/v) in *n*-butanol was used to locate the sugars. In some cases glycosides were eluted with warm water from the paper, hydrolysed with N-H_2SO_4 for 3 hr. at 100°, neutralized with $BaCO_3$ and rechromatographed for the identification of the sugar components.

Plant extracts. Extracts were prepared as described by Yemm & Willis (1954). Leaves were exhaustively extracted with 70% (v/v) ethanol and the extracts evaporated to dryness *in vacuo*. They were then taken up in warm water and cleared with aluminium hydroxide.

RESULTS

The course of the colour reaction. With different sugars the intensity of colour was measured after different periods of heating, and the results for hexoses, 6-deoxyaldohexoses and aldopentoses are shown in Figs. 1–3. Of the hexoses, the ketoses fructose and sorbose react most quickly giving maximum colour development after about 1·5 min. and thereafter a marked decrease in colour with further heating. The rapid reaction with ketoses has been exploited by Johanson (1953*b*) to give a specific colour test for these sugars. The aldoses react more slowly and give a much lower colour production which reaches a maximum after about 6–7 min. for galactose and mannose and about 10 min. for glucose. It is clear that the rate of decrease of colour is approximately the same for both ketohexoses and aldohexoses. A solution of sucrose (calculated as invert sugar) gave colour densities agreeing closely with those expected from a mixture of equal parts of glucose and fructose, when the necessary correction for the non-linear calibration of the colorimeter had been made. 6-Deoxyaldohexoses (rhamnose and fucose) were very similar with regard to colour production and destruction (Fig. 2); for both sugars colour was developed more slowly than for ketoses and maximum absorption was observed after 3 min. heating. The aldopentoses tested, xylose, ribose and arabinose, produced much less colour than the hexoses or 6-deoxyaldohexoses. All three aldopentoses showed rapid colour production which reached a maximum after heating for about 2–2·5 min. (cf. Trevelyan & Harrison, 1952), and then the blue-green colour very quickly disappeared, there being only little absorption after 10 min. heating. At the time of maximum absorption xylose gave a much more intense colour than either ribose or arabinose. The initial rate of colour destruction was very much greater than that of the hexoses, the 6-deoxyaldohexoses being intermediate in this respect. In view of the rapid disappearance of colour with pentoses, the reaction was investigated at lower temperatures with xylose (Fig. 4). Progressive improvement in the stability of the colour was obtained, but the maximum absorption was substantially reduced at lower temperatures. Similar results were obtained with arabinose.

Absorption spectra. For several of the sugars detailed absorption spectra were investigated, both at the time of maximum colour production and after more prolonged reaction. The results are shown in Fig. 5*A*, *B*. With pentoses and hexoses the maximum optical density is at approximately 630 mμ., and is slightly displaced in the case of rhamnose. On more prolonged heating the characteristic absorption band of arabinose disappeared

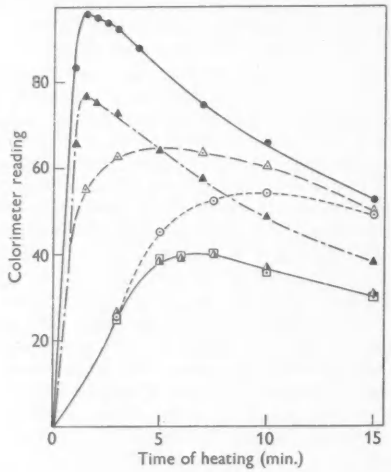

Fig. 1. Colour production during anthrone reaction with hexoses. Colour measurements are corrected for reagent blanks; 75 μg. of each sugar in 1 ml. of water was added to 5 ml. of anthrone reagent. ●—●, D-Fructose; ▲-·-▲, L-sorbose; ⊙---⊙, D-glucose; ▲—▲, D-galactose; ⊡—⊡, D-mannose; △---△, sucrose (calculated as invert sugar).

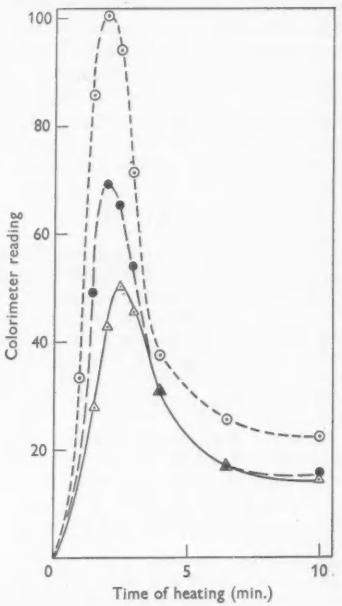

Fig. 3. Colour production during anthrone reaction with aldopentoses. Reaction conditions and colour measurements as for Fig. 1, except that 300 μg. of sugar in 1 ml. of water was added to 5 ml. of anthrone reagent in all tests. ⊙---⊙, D-Xylose; ●---●, D-ribose; △—△, L-arabinose.

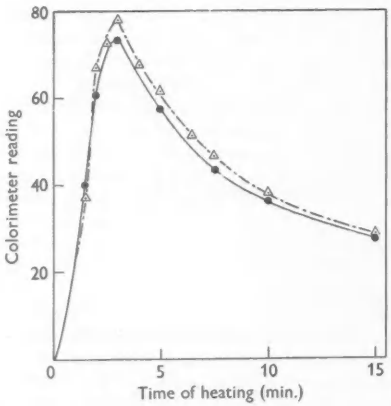

Fig. 2. Colour production during anthrone reaction with 6-deoxyaldohexoses. Reaction conditions for each sugar (75 μg./ml.) and measurements as for Fig. 1. △-·-△, L-Rhamnose; ●—●, L-fucose.

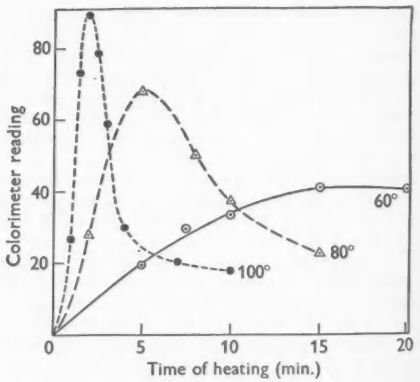

Fig. 4. The effect of temperature on the anthrone reaction with D-xylose. The pentose (240 μg. in 1 ml. of water) was added to 5 ml. of anthrone reagent and allowed to react in a boiling-water bath (●---●), at 80 $\pm$ 1° (△---△) and at 60 $\pm$ 1° (⊙—⊙). The readings are corrected for reagent blanks.

and there was an increase of optical density in the region of 500 mμ. Essentially similar changes, although less pronounced, occurred with hexose sugars.

Analysis of sugar mixtures. When mixtures of sugars were examined it was observed that the absorption curves were consistent with those expected from a summation of the individual sugars. In view of the findings of Johanson (1953*a*) a special test was made with mixtures of glucose and arabinose. The absorption spectra of the individual sugars and of a mixture of the two after 10 min. heating are shown in Fig. 5*C*. At all the wavelengths investigated the optical density of the sugar mixture agreed satisfactorily with that of the sum of the individual sugars, and there was no evidence of appreciable interaction such as that described by Johanson (1953*a*).

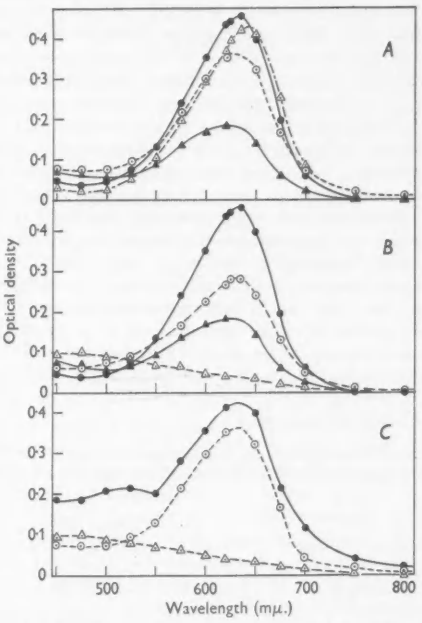

Fig. 5. Absorption spectra of pure sugars and their mixtures after varying periods of heating with anthrone reagent. Measurements of optical density are corrected for reagent blanks. *A*. Pure sugars after optimum heating period. ●—●, D-Fructose (35 μg.; 3 min. heating); △·-·△, L-rhamnose (40 μg.; 3 min. heating); ⊙- - -⊙, D-glucose (50 μg.; 10 min. heating); ▲—▲, L-arabinose (150 μg.; 3 min. heating). *B*. The effects of prolonged heating. D-Fructose (35 μg.): ●—●, 3 min. heating; ⊙- - -⊙, 10 min. heating. L-Arabinose (150 μg.): ▲—▲, 3 min. heating; △- - -△, 10 min. heating. *C*. Absorption spectra of glucose, arabinose and their mixture after 10 min. at 100°. △- - -△, L-Arabinose (150 μg.); ⊙- - -⊙, D-glucose (50 μg.); ●—●, L-arabinose (150 μg.) + D-glucose (50 μg.).

More complex mixtures of sugars have been tested with both the spectrophotometer and the colorimeter. The results of one such test carried out with arabinose, rhamnose, fructose and glucose and a mixture of these sugars are given in Table 1. It will be seen that the optical density of the mixture agreed within ±3% of the sum of the optical densities of the individual sugars after heating periods of 3 and 10 min. Several mixtures of hexoses, 6-deoxyhexoses and pentoses have also been investigated by means of the colorimeter and in all cases satisfactory agreement with the expected sum was observed.

The estimation of pure sugars and their recovery from plant extracts. The effective ranges of sugar concentration over which the method described here can be used are indicated in Table 2, together with the heating times adopted for analysis of different sugars. The reproducibility of colour production has been judged from a number of calibrations carried out with different samples of reagent over several months. The results for glucose, fructose and sucrose are based on more than ten determinations;

Table 1. *Reaction of anthrone with sugars and sugar mixture*

In each test the sugar (or mixture) in 1 ml. of solution was added to 5 ml. of anthrone reagent and the optical density measured after heating at 100° for 3 and 10 min.

Sugar	Weight of sugar (μg.)	Optical density at 620 mμ. 3 min.	10 min.
L-Arabinose	51·1	0·064	0·012
L-Rhamnose	22·8	0·204	0·095
D-Fructose	24·8	0·310	0·193
D-Glucose	25·0	0·074	0·169
Mixture of above	123·7	0·635	0·485

Table 2. *The range of sugar concentration and reproducibility of colorimeter readings*

The maximum variation was estimated from calibrations made on different occasions and calculated as a percentage of the colorimeter reading for the middle of the effective range.

Sugar	Time of heating (min.)	Effective range (μg./ml.)	Max. variation (±%)
D-Glucose	10	10–100	3·8
D-Fructose	10	10– 80	2·0
Sucrose	10	10– 80	3·2
L-Sorbose	10	10–100	2·0
D-Galactose	10	10–125	2·8
D-Mannose	10	10–125	3·4
L-Rhamnose	3	10– 70	2·0
L-Fucose	3	10– 70	2·0
D-Xylose	3	30–300	10·7
D-Ribose	3	35–350	4·5
L-Arabinose	3	40–400	5·0

more limited data are available for other sugars. The maximum variation in calibration did not exceed ±4% except for pentoses, where, owing to the instability of the colour complex, the heating and cooling conditions are of critical importance. It has been observed consistently that a higher degree of reproducibility can be obtained in a series of analyses carried out with the same solution of anthrone. For example, replicate estimates made in this way with a solution of glucose containing 50 μg./ml. had a standard error of ±0·30 μg./ml., with a maximum variation of ±1·6%, and at a concentration of 100 μg./ml., the standard error was ±0·58 μg./ml. and maximum variation ±1·4%. It has therefore been our general practice to include at least one determination of a known amount of sugar in each series of analyses. One important source of error arises from the variation shown in blank determinations, and as this variation appears to be largely due to contamination by dust, care in cleaning glassware is essential.

The effect of a number of compounds on colour production has been examined by Scott & Melvin (1953), and, with the exception of chlorides, only little interference was found. Increased colour production of sugars in the presence of chloride has been confirmed in this laboratory; when solutions containing sodium chloride are used, calibration with pure sugars with the same salt concentration is required.

The reliability of the anthrone method for the analysis of plant extracts has been tested by determining the recovery of sugars added to a leaf extract. The results of this investigation are given in Table 3. Satisfactory recovery of all the sugars tested was obtained. As found above, the greatest variation was observed when arabinose was added, in all probability owing to the instability of the anthrone colour complex with this sugar.

The analysis of plant extracts. A further test of the anthrone method has been made by comparing it with a copper reagent (Somogyi, 1945) for the determination of soluble carbohydrate in leaf tissues. It has already been shown (Yemm & Willis, 1954) that for extracts of leaves of Shasta daisy (*Chrysanthemum maximum*) estimations by the anthrone method agree substantially with those by means of copper reagent, after correction has been made for the substances not removed by rapid fermentation by yeast. Similar agreement has now been found for extracts of leaves of broad bean (*Vicia faba*) and here it is highly probable that the well-defined anthrone reaction obtained with leaf extracts after fermentation is due to the presence of stable glycosides. It was found chromatographically that sucrose, glucose and fructose were the only detectable free sugars in the leaves, but that pigment glycosides were also present. After hydrolysis of one of these pigments, obtained by elution from paper, approximately equal quantities of rhamnose and arabinose together with small amounts of glucose were detected on chromatograms. Support for this finding was obtained from a more detailed study of the course of the anthrone reaction with the carbohydrates remaining in the leaf extract after fermentation. Colour developed to a maximum after about 3 min. heating and then declined to a value of about 64% of its maximum

Table 3. *Recovery of sugars added to leaf extract*

Weighed quantities of sugars were added to an extract of broad bean leaves. The initial sugar content of the leaf extract was calculated as a mixture of equal parts of glucose and fructose, and the recovery of added sugars estimated from calibration graphs for the individual sugars.

Sugar added to extract	Time of heating (min.)	Wt. of sugar added (μg./ml.)	Total sugar found (μg./ml.)	No. of observations	Mean recovery and max. variation (%)
None	3	—	36·0±0·9	4	—
	10	—	38·6±0·3	4	—
Sucrose	10	40	78·5	3	99·7±1·4
Glucose	10	40	79·1	3	101·2±3·2
Fructose	10	40	78·8	3	100·5±2·6
Rhamnose	3	40	76·1	5	100·3±2·1
Arabinose	3	150	187·0	4	100·7±4·7

Table 4. *Analysis of extracts of broad bean leaves with anthrone*

These estimates, made by heating 1 ml. of extract with 5 ml. of anthrone reagent for 10 min., are expressed as equivalent mg. glucose/g. dry wt.

Sample no. (collected 18. vii. 53)	Time collected (G.M.T.)	Epidermis		Mesophyll	
		Free sugars	Glycosides	Free sugars	Glycosides
1	2 a.m.	31·5	35·7	51·7	19·6
2	10 a.m.	51·3	38·4	71·5	18·1
3	5.30 p.m.	84·1	35·8	106·9	18·1

after 10 min. heating. Both the formation and destruction of colour are consistent with the reaction of a mixture of sugars containing mainly rhamnose and arabinose. The analytical results suggest that in certain tissues of the leaf these glycosidic pigments may constitute a considerable part of the total soluble carbohydrate. Some typical results are summarized in Table 4. In the sample collected during the night the major part of the carbohydrates reacting with anthrone in extracts of the epidermis is derived from glycosides, and, calculated as equal amounts of arabinose and rhamnose, would constitute about 75% of the total carbohydrate of the extract. Despite large fluctuations in the other carbohydrates (mostly sucrose) during the day, the glycoside carbohydrates remain relatively constant in both the epidermis and the mesophyll.

DISCUSSION

As pointed out by Sattler & Zerban (1948), it is very probable that the reaction between sugars and anthrone depends on dehydration and ring formation to form furfural or furfural derivatives which may then react with anthrone. Evidence of these changes in polysaccharides and sugars heated with strong sulphuric acid has been given by Black (1951) and by Love (1953) from measurements of ultraviolet absorption. There is a general parallel between the rate of formation of 5-hydroxymethylfurfural from different hexoses (Love, 1953) and the rate of development of the anthrone colour as shown in Fig. 1. Furthermore, the relative colour yield with anthrone from different hexoses generally corresponds to the amounts of hydroxymethylfurfural formed as judged by Love's absorption measurements at 320 mμ. The parallel seems to hold also for the production of methylfurfural from rhamnose, although here the destruction of the colour during heating is somewhat greater than with other hexoses. With pentoses, on the other hand, the yield of colour is much less than with hexoses, and this can be related to the very high rate of destruction under the conditions normally employed for the reaction. Xylose gives the most intense colour (Fig. 3), corresponding to the most rapid and greatest yield of furfural (Love, 1953). However, the low colour yields of arabinose relative to ribose do not appear to reflect the amounts of furfural produced from these sugars. It is clear that the anthrone method as applied to pentoses is seriously limited by the stability of the furfural–anthrone complex. Although the stability is greater at lower temperatures the sensitivity of the reagent progressively decreases, so that little advantage can be gained by carrying out the reaction at 60° or 80° (Fig. 4). It seems unlikely, therefore, that the anthrone reaction with different hexoses and pentoses can be fully interpreted in terms of the yield and stability of their respective furfural derivatives, especially since furfural and methylfurfural are considerably more stable than hydroxymethylfurfural in acid solution (see Newth, 1951).

For the estimation of soluble sugars in plant materials, a reaction period of 10 min. at 100° has several advantages, and has been adopted for routine analysis of tissue extracts. Under these conditions glucose and fructose, which together make up the bulk of the hexoses, give approximately the same colour density so that fairly accurate estimates of total sugars can be obtained without separate determination of the constituents. Pentoses do not interfere seriously in these estimations; even if present in equal amounts with hexoses the colour density produced by the pentose at 620 mμ. would represent only about 10% of that of the hexose alone. No evidence has been found of interaction of pentoses and hexoses of the type described by Johanson (1953*a*), and it seems possible that the discrepancy is due to different experimental conditions. In the present work with both a disaccharide (sucrose) and artificial mixtures of monosaccharides the colour reaction has been shown to agree closely with that expected from the behaviour of the individual sugars. Similar findings with lactose and maltose have been described by Morris (1948), who further showed that glycogen behaved in the same way as its hydrolysate. Purified starches have also been found, in this laboratory, to react in agreement with their equivalent of glucose. The method has therefore been shown to give reproducible results with a wide range of hexoses and with polysaccharides. However, as a test for pentoses the reaction is much less sensitive, although satisfactory estimates can be made with carefully standardized heating periods of 2–3 min. A useful feature of the method is that some information may be gained regarding the type of carbohydrate present in extracts under test by studying the course of the anthrone reaction.

The high specificity of the anthrone reaction for carbohydrate (Morris, 1948; Sattler & Zerban, 1948) makes it particularly useful for the analysis of tissue extracts. It has been shown in the present work that, under the routine conditions employed, uronic acids give a small but appreciable colour formation. For example, D-galacturonic acid gives about 10% of the colour produced by an equal weight of glucose after 10 min. reaction. In connexion with other investigations it has been established that lactic acid and glycerol do not produce green colours with anthrone, but that, in agreement with Shetlar (1952), carbohydrates in the presence of tryptophan give low optical densities

at 620 mμ. Under our conditions, 50 μg. of glucose with 40 μg. of DL-tryptophan gives a colour density of about 95% of that of the sugar alone.

SUMMARY

1. The anthrone reaction has been investigated with a range of naturally occurring hexoses and pentoses.

2. The course of colour production varies widely with different sugars, and the test is much less sensitive for pentoses than for hexoses.

3. Sugar mixtures give results agreeing closely with those expected from the colour production of the individual sugars. Under the conditions used here there is no evidence of serious interaction of hexoses and pentoses.

4. For the estimation of soluble sugars in plant extracts the method yields results comparable with those obtained with the copper reagent, but includes the sugars of stable glycosides which may constitute a large proportion of the soluble carbohydrates in some plant tissues. Used in conjunction with chromatographic identification, the anthrone method is of particular value when limited quantities of tissue are available.

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Separation of the Bile Pigments of Serum, Bile and Urine

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Van den Bergh & Müller (1916) described two types of diazo reaction for bile pigment in serum. The sera of normal persons, and of patients with haemolytic jaundice, gave a red colour with diazotized sulphanilic acid only in the presence of ethanol. This was called an 'indirect' reaction, and was distinguished from the 'direct' reaction of bile, or sera from patients with obstructive jaundice, for which ethanol was unnecessary. The direct- and indirect-reacting components of serum were separated chromatographically by Cole & Lathe (1953). While the indirect-reacting pigment appeared to be bilirubin, it was suggested that the direct-reacting fraction was a mixture of two pigments. This has now been confirmed by separating these pigments on reverse-phase chromatograms. These pigments have also been found in human necropsy bile, and in the urine of patients with obstructive jaundice.

METHODS

Extraction of pigments. Pigments were prepared from the sera of jaundiced patients by adding, with stirring, to 1 vol. of serum, 0·18 vol. of saturated $(NH_4)_2SO_4$ and 2·5 vol. of ethanol. After standing for 30 min. the precipitate was removed by centrifuging. The supernatant was stored at $-12°$ and was used as soon as possible, since the polar pigments were found to be altered after storing for several days at this temperature. Before use the supernatant was taken to dryness *in vacuo* at $<40°$.

The pigments were separated from specimens of bile by the addition of 70 g. of $(NH_4)_2SO_4$/100 ml. of bile. After vigorous mechanical stirring, the mixture was centrifuged and the resulting pellet of protein, pigment, etc., was lifted from the surface of the solution. It was stored in this form at $-70°$. Pigments and lipids were separated from the protein and salt of the pellet by grinding it with *n*-butanol: methanol (1:1, v/v) and re-extracting until an almost colourless solution was obtained. This procedure was then

repeated several times with 50% aqueous methanol until the extracting fluids became turbid. The residue, which was still pigmented, was discarded. The extracts were taken to dryness *in vacuo* at a temperature of <40°.

The urine of patients with obstructive jaundice (24 hr. samples) was collected over $CHCl_3$, at room temperature. The urine, while being stirred mechanically, was saturated with $(NH_4)_2SO_4$ (700 g./l.); during stirring, *n*-butanol was added to a final concentration of 1–2%. The turbid solution was then transferred to a separating funnel where the pigmented precipitate rose to the surface. After standing for about 1 hr. most of the lower liquid was drained off, the remainder was centrifuged, and the relatively compact pellet was removed. If a small amount of deeply coloured butanol separated it was taken off with the pellet. The whole was kept at −70° until required. The pigments were extracted from the urine deposit in the same way as from bile concentrates.

Chromatography. Reverse-phase chromatograms were set up in 16 mm. (internal diameter) glass tubes using silicone-treated kieselguhr (prepared with Silicone Fluid M441 of Imperial Chemical Industries Ltd.) as described by Howard & Martin (1950). The following solvent systems were used: *A*—CCl_4, 25 vol.; $CHCl_3$, 25 vol.; CH_3OH, 38 vol.; 0·025 M phosphate buffer pH 6, 12 vol. *B*—*n*-butanol, 50 vol.; 0·005 M phosphate buffer pH 6, 50 vol. *C*—acetone, 25·9 ml.; $(NH_4)_2SO_4$, 17·0 g.; 0·05 M phosphate buffer, pH 6, 62·5 ml. (sufficient 2·5 N-NaOH, about 1·7 ml., was added to restore the pH to 6). Unless stated otherwise, all buffers were prepared as described by Cole (1933).

Columns with solvents *A* and *B* were made with 6 g. of kieselguhr and 3 ml. of stationary phase, while for system *C* half these amounts were used. All columns were run at room temperature (18–25°). The solvent was eluted from columns at a rate of 1 ml./3 min. (except for solvent *C*, 1 ml./10min.). The depth of colour in the eluates was read in the microcell of a Spekker absorptiometer using Ilford filters no. 601 (maximum transmission about 430 mμ.).

Diazotization. The reagent was prepared by mixing 10 ml. of 0·1% (w/v) sulphanilic acid in 0·25 N-HCl with 0·3 ml. of 0·5% (w/v) $NaNO_2$. Half a vol. of this solution was added to 1 vol. of the solution to be diazotized, followed by 1·5 or 4·5 vol. of ethanol, depending on the anticipated depth of colour. Extinctions were read in a 10 mm. cell using an Ilford green filter (no. 404 with maximal transmission at about 530 mμ.). Determination of the 'directness' of the diazotization was made on the aqueous layer after removal of *n*-butanol from the eluates by shaking them with 8 to 10 vol. of light petroleum.

For absorption curves of the azo compounds of pigments obtained from chromatographic columns, 0·2 ml. of diazo reagent was added to 3 ml. of the eluate, together with 0·3 ml. 3·6% $Na_2HPO_4, 12H_2O$ and ethanol (1·0 and 0·5 ml.) for columns using solvent systems *A* and *B* respectively. The rate of colour development under these conditions was observed and the absorption curves were plotted when the colour was maximal, using a Beckman quartz spectrophotometer.

Ultraviolet and visible absorption. A Unicam quartz spectrophotometer was used, with 10 mm. quartz cells. Turbidity was prevented in eluates from butanol columns by the addition of 0·2 ml. ethanol to 4 ml. of eluate. Readings were made generally at intervals of 10 mμ. (2 mμ. near peaks).

Solvents. *n*-Butanol, 'Laboratory Grade' and AnalaR grades of all other solvents were used without further purification.

Standards. A number of commercial samples of bilirubin were examined chromatographically with solvent system *A*. In each case one band of pigment predominated, though the amount of other pigments varied from specimen to specimen. Bilirubin from British Drug Houses Ltd., which contained only small amounts of these other pigments, was used as a standard in this work, and only the main pigment band on the column has been used for absorption studies.

RESULTS

Chromatographic separation of direct-reacting pigments. Cole & Lathe (1953) used solvent system *A*, on water-repellent kieselguhr, for the chromatographic separation of the bile pigments of serum into two components. In this system the polar materials sometimes appeared as two bands but these were always eluted together. For convenience these two polar pigments are called pigment I (the less polar) and pigment II (the more polar).

The polar pigments I and II can be separated on silicone-treated kieselguhr, using solvent mixture *B*. In Fig. 1, a chromatogram of serum from a case of hepatitis shows that in this system the more polar pigment II moved well ahead of the less polar, pigment I, which had an *R* value of about 0·3. Bilirubin and biliverdin had *R* values of 0 and 0·15, respectively, in this system.

The rate of movement of pigment II on this column was rather variable, possibly due to temperature changes, and usually it moved close to the solvent front (*R* value 0·7–0·95). Several more polar systems were tried in an endeavour to retard the pigment. Of these systems *C* was the most satisfactory, although the *R* value of pigment II was still rather variable, and tailing was apparent.

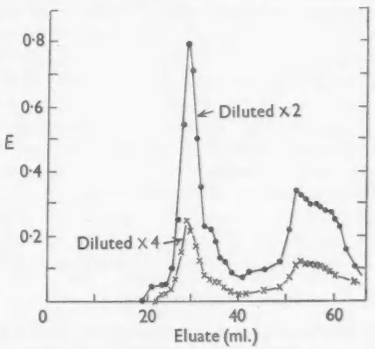

Fig. 1. Elution of polar pigments of icteric serum from chromatogram (solvent system *B*), showing depth of colour (●—●) and concentration of diazo-reacting material (×—×). Details in text, this page.

Absorption curves of serum pigments. The spectral absorption curves of serum pigments, and of a bilirubin standard, were determined in the solvent as they were eluted from the chromatography columns. In Fig. 2 (solvent system *A*) bilirubin is compared with the non-polar pigment obtained from the serum of a patient with haemolytic jaundice, and with a band of polar pigments from the serum of a patient with obstructive jaundice. Bilirubin and the non-polar pigment of serum share the same peak at 454 mμ., while the curve of the polar pigments has a peak at 419 mμ. A part of the ultraviolet absorption of this latter curve was due to contaminants, since it could be demonstrated in eluates prepared in an identical way from normal unpigmented serum. Contaminants may also have made a minor contribution to the ultraviolet absorption of the non-polar serum pigment.

The absorption curves of pigments I and II were compared after elution from a column using solvent system *B* (Fig. 3). The absorption curve of bilirubin, which is included here, was not obtained as an eluate, since bilirubin does not move on this column. The bilirubin (from a specimen known to be pure by its chromatographic behaviour) was dissolved in the dilute alkaline component of the pH 6 buffer of system *B*, together with the amount of *n*-butanol necessary to saturate it, and then the acid component of the buffer was added. The absorption maxima of bilirubin, pigment I, and pigment II were at 450, 452 and 448 mμ. respectively. Absorption curves were also obtained from chromatographed extracts of normal unpigmented serum. Those eluates which corresponded with pigment I had negligible ultraviolet absorption, while the more polar eluates, comparable with pigment II, showed considerable ultraviolet absorption.

Diazotization of pigments. The non-polar pigment of serum was known previously to give no diazo reaction in the absence of ethanol. In the present study, both pigments I and II gave an immediate, 'direct', reaction when diazotized in the absence of ethanol. The rate of colour development in the presence of ethanol was the same for all three pigments, as was the relationship between the amount of yellow colour and the amount of azo compound formed. The products of diazotization of all these pigments had maximal absorption at 526 mμ., at pH 4. The shape of the absorption curves was similar above 450 mμ., and changes in pH resulted only in a shift of the peak. Below 450 mμ. absorption depended partly on the amount of original reactants present.

Pigments in bile and urine. The extracts of pigment from the urines of a number of patients with obstructive jaundice have been examined chromatographically. Some pigments I and II and small quantities of bilirubin were found. Pigment I was the more abundant of the polar pigments. Frequently there were larger amounts of a red-brown pigment, running slightly faster than pigment I on the column using solvent *B*.

Bile extracts had substantially larger proportions of the polar bile pigments than had urine extracts, and pigment II was more abundant than pigment I.

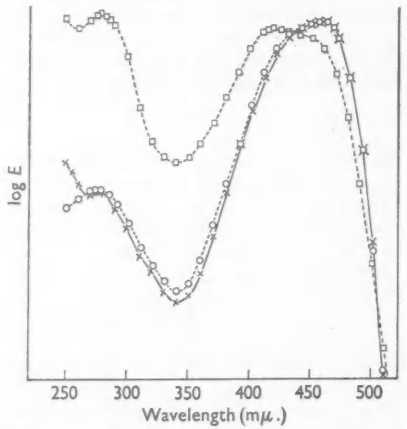

Fig. 2. Comparative absorption spectra of bilirubin (○- - - -○), the non-polar pigment of icteric (haemolytic) serum (×—×), and the polar pigments (□- - - -□) from icteric (obstructive) serum. The extinctions shown for the non-polar and polar pigments have no strict quantitative significance and are only of a comparative character.

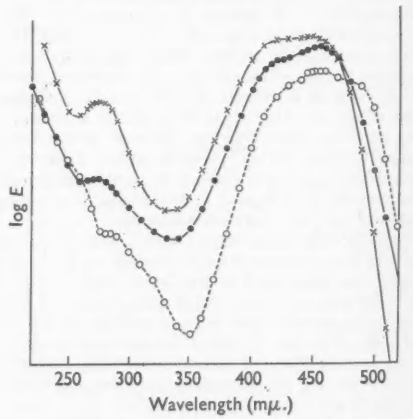

Fig. 3. Comparative absorption spectra of pigment I (●—●) and pigment II (×—×), as eluted from a chromatogram of solvent system *B*, and of bilirubin (○- - - -○) dissolved in the polar phase of solvent system *B*. For significance of the extinctions of pigments I and II see Fig. 2.

Bilirubin accounted for only a small proportion of the material giving a positive diazo reaction. Moreover, there were other pigments than bilirubin, pigments I and II, which gave this reaction.

DISCUSSION

Cole & Lathe (1953) suggested that the non-polar pigment of serum was bilirubin, basing this on the identical behaviour of these two substances on the chromatogram and in the direct and indirect diazo reactions. Additional evidence is now provided by a comparison of their adsorption curves (Fig. 2). The main difference is in the ultraviolet range. However, the increased ultraviolet absorption shown by eluates containing the non-polar serum pigment is attributable to other materials which are present in normal serum, and which are eluted from the column in the same position as bilirubin. This feature of the ultraviolet absorption has also been noted by Claireaux, Cole & Lathe (1953) in similar fractions of jaundiced brain. Throughout the visible range, on the other hand, the absorption curves of the non-polar serum pigment and of bilirubin are almost identical. Thus, although not entirely conclusive, all the evidence points to bilirubin as the non-polar pigment of serum.

No specimen of mesobilirubin has been available for comparison with the serum pigments. However, its maximal absorption (in chloroform), 25 mμ. closer to the ultraviolet, distinguishes it from bilirubin (Heilmeyer, 1943), and therefore from the non-polar serum pigment. On the other hand, mesobilirubin is unlikely to be one of the polar pigments, since Feruglio & Salvini (1947) have reported that mesobilirubin is indistinguishable from bilirubin in the diazo reaction. In its chromatographic behaviour mesobilirubin would be expected to be less, rather than more, polar than bilirubin, because it lacks two double bonds in the β substituents, and is known to be more soluble in chloroform than is bilirubin. In addition, mesobilirubin is more stable than the polar pigments. It seems probable then that mesobilirubin is not one of the serum bile pigments.

The component of serum bile pigment which reacts directly in the diazo test, and is prominent in the sera of patients with obstructive jaundice, can be separated into two pigments (I and II), both of which react directly. Considerable variations in the relative proportions of these two polar pigments have been noticed.

The two polar pigments are labile and have not been isolated in pure form. However, following chromatography they appear to be relatively free from other materials which absorb in the visible range. The spectral curves of pigments I and II, and bilirubin are all similar, though distinctive features are noticed. Differences between the spectra of bilirubin and the polar pigments are noted in the polar phase of solvent *A* (Fig. 2), the peak of the polar pigments being 25 mμ. towards the ultraviolet. The ultraviolet peak, in the region of 275 mμ., seems to be most prominent in pigment II, in solvent *B*. This absorption peak is found in the corresponding eluates of normal serum, and may be due to contaminants. Although these materials are impure it seems probable from the similarity of their absorption curves, and of those of their diazo compounds, that they have the same tetrapyrrole structure as bilirubin. Whatever changes occur when the liver transforms bilirubin into pigments I and II, it does not greatly affect the chromogenic structure.

It has been known for a long time (van den Bergh, 1918; Andrewes, 1924) that direct-reacting sera or urines become indirect-reacting, like bilirubin, if they are allowed to stand at room temperature. It has been our experience that even relatively mild treatment of the polar pigments is likely to lead to the appearance of a band of bilirubin in a subsequent chromatogram. Moreover, a transformation of pigment I into pigment II and bilirubin can be produced by warming and evaporation. The reverse reactions have not been observed, and it has not been possible to change bilirubin into the polar pigments in experiments *in vitro*. Until more is known about their interrelation it is not possible to state how much of pigment I or pigment II may be produced from the other during manipulation. It is certain that bilirubin will be observed as an artifact after rigorous treatment of bile, and of some urines and sera.

It has been stated frequently that the main pigment of human bile is bilirubin. This now requires qualification. In earlier studies the preliminary treatment of the bile was probably sufficient to produce bilirubin from the polar pigments. The procedures outlined here preserve substantial amounts of these two pigments, and bilirubin occurs in only minor amounts. Other bile pigments have also been observed, and some of them gave a diazo reaction. Only three naturally occurring pigments have previously been reported to give the diazo reaction, namely, bilirubin, mesobilirubin and mesobilene-*a* or dihydromesobilirubin (Siedel, 1951). It seems probable that this series could be extended, or, alternatively, that in bile there occur a number of unstable pigments which are transformed to those giving a positive reaction.

SUMMARY

1. A study of the absorption curve of the non-polar bile pigment, which predominates in the serum of patients with haemolytic jaundice, has provided additional evidence that it is bilirubin.

2. The serum of patients with obstructive jaundice has been shown to contain two pigments

reacting 'directly' in the van den Bergh test. Their absorption curves are similar, though not identical, and they differ from that of bilirubin.

3. The visible absorption curves of the azo compounds of these three pigments are indistinguishable.

4. The two direct-reacting pigments of serum occur in small amounts in the urine of patients with obstructive jaundice, and in large quantities in gallbladder bile obtained at necropsy, where they are more abundant than bilirubin.

5. The proportion between the two direct-reacting pigments of serum is variable.

6. There is evidence that other diazo-positive pigments occur in bile and urine.

We wish to express our thanks to the many clinical colleagues who have provided us with sera, and to Dr W. Hunt, St Mary's Hospital, Paddington, for obtaining specimens of autopsy bile. A grant from the Medical Research Council is also acknowledged by one of us (B. H. B.).

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Polyol Dehydrogenases

1. THE SPECIFICITY OF RAT-LIVER POLYOL DEHYDROGENASE

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The 'sorbitol' dehydrogenase of rat liver (Blakley, 1951) reacts with diphosphopyridine nucleotide (DPN) to catalyse the reversible oxidations:

$$\text{D-sorbitol} + \text{DPN}^+ \rightleftharpoons \text{D-fructose} + \text{DPNH} + \text{H}^+,$$

$$\text{L-iditol} + \text{DPN}^+ \rightleftharpoons \text{L-sorbose} + \text{DPNH} + \text{H}^+.$$

Finding that glucose, gluconate, mannitol, dulcitol and D-iditol did not react with the enzyme, Blakley (1951) concluded that an oxidizable substrate must possess a primary alcohol group at C-1 adjacent to the site of oxidation (C-2) and a stereochemical pattern at C-2 and C-4 identical with that occurring in sorbitol and L-iditol.

Blakley's evidence eliminated the configuration at C-5 as a determinant of specificity but did not decide the importance of the configuration at C-3. In order to settle this point, and to gain more knowledge of the enzyme, we have investigated the oxidation of a wider range of polyhydric alcohols.

MATERIALS AND METHODS

Enzyme. The enzyme solution was the dialysed preparation *B* of Blakley (1951).

Coenzymes. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were products of the Sigma Chemical Co., St Louis, Missouri. The former was found to be 65% pure by the LePage (1947) assay and the latter 60% pure (assayed with glucose 6-phosphate dehydrogenase).

Acyclic polyols. Xylitol (m.p. 92–93·5°) and D-arabitol (m.p. 102°) were prepared by catalytic reduction of D-xylose and D-arabinose respectively, according to Carson, Waisbrot & Jones (1943). Allitol (m.p. 148–149°) was prepared by oxidation of divinyl glycol (Lespieau, 1946). Erythritol, ribitol and L-arabitol were obtained from Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A. Sorbitol monohydrate, D-mannitol and dulcitol were products (C.P. grade) of the Pfanstiehl Chemical Co., U.S.A. The following were gifts from Dr N. K. Richtmyer, National Institutes of Health, Bethesda, U.S.A.: D-gulitol, 6-deoxy-L-gulitol, D-talitol, volemitol, perseitol, β-sedoheptitol and L-*gulo*-D-*gala*-heptitol.

Cyclitols. Scyllitol (m.p. 347°) was prepared from scyllitol hexaacetate according to Posternak (1942). *meso*Inositol (C.P. grade) was obtained from the Pfanstiehl Chemical Co.; *d*- and *l*-inositol, *l*-viburnitol and scyllitol hexaacetate were gifts from Mrs E. Smith, Chemistry Department, University of Sydney.

Sugars. The sugars used were obtained from the following sources: D-xylose (C.P. grade), Pfanstiehl Chemical Co.; D-arabinose, Nutritional Biochemicals Inc.; D-*manno*heptulose and D-*gluco*heptulose, General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.; perseulose hemihydrate and

sedoheptulosan monohydrate (gifts), Dr N. K. Richtmyer; D-xylulose (gift) prepared from the crystalline monoacetone derivative according to Levene & Tipson (1934) by Mr R. G. Kulka; D-tagatose (gift) prepared according to Reichstein & Bosshard (1934) by Mr D. R. D. Shaw. Sedoheptulose: an equilibrium mixture containing approximately 20% of sedoheptulose and 80% of sedoheptulosan was prepared by heating (steam bath) a solution of sedoheptulosan in N-H_2SO_4 for 3 hr. and removing the acid by passage through an anion-exchange resin (Amberlite IR 4-B).

Analytical methods

Oxygen consumption at 38° was measured by the conventional Warburg manometric technique. In all experiments methylene blue was added in order to link the dehydrogenase system to molecular oxygen.

Reduction and re-oxidation of DPN. Oxidation and reduction of the coenzyme were followed by measuring optical density at 340 mμ. in a Beckman DU spectrophotometer. The reaction mixtures were contained in quartz cuvettes having a 1 cm. light path.

Reducing sugar was determined according to Somogyi (1945*a*) after deproteinization with $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1945*b*), and removal of methylene blue. Much of the methylene blue was adsorbed on the protein precipitate and the residue was removed by treatment of the protein-free filtrate with the minimum amount of Merck's activated charcoal.

Detection of ketoses. After removal of protein and methylene blue (as above) ketoses were identified by the resorcinol test using the reagents of Bacon & Bell (1948), the carbazole reaction (Dische & Berenfreund, 1951) and the isolation of phenylosazones. The ketoses were distinguished in the carbazole reaction by observation of the colour developed initially and by plotting absorption spectra in the wavelength range 440–630 mμ., maximum absorption being at 560 mμ. for ketohexoses and at 538 mμ. for ketopentoses (see Dische & Berenfreund, 1951). In the resorcinol test the colours developed were green (ketopentose), cherry red (ketohexose) and purplish red (ketoheptose), and the respective absorption curves in the visible region of the spectrum agreed with those which have been determined by Mr R. G. Kulka (unpublished observation) for pure sugars. The individual heptuloses were identified by paper chromatography with a solvent mixture composed of *n*-butanol, ethanol and water (Bevenue & Williams, 1951).

Optical rotations were measured in a standard Hilger polarimeter reading to ±0·01°.

Preparation of phenylosazones

When the scale of an experiment was limited by the amount of polyol available, the osazones were prepared from the contents (deproteinized and cleared of methylene blue) of Warburg flasks. When there was no such restriction the enzyme solution (about 100 ml.) containing polyol (0·048 M), DPN ($9{\cdot}6 \times 10^{-5}$ M), potassium phosphate buffer, pH 7·9 (1×10^{-3} M) and methylene blue ($9{\cdot}5 \times 10^{-4}$ M) was divided into six equal parts and shaken in stoppered 50 ml. conical flasks for 12 hr. at 38°. The gaseous phase was air. Oxygen uptake was followed manometrically in a pilot Warburg flask containing a 3 ml. sample of the reaction mixture. The combined solutions were deproteinized either with $HClO_4$ according to Neuberg, Strauss & Lipkin (1944) or with 0·1 vol. of 7% (w/v) metaphosphoric acid. Residual methylene blue was removed with Merck's activated charcoal, and after neutralization with K_2CO_3 the filtrate was concentrated at room temperature *in vacuo* over H_2SO_4 until its volume was reduced to 30–40 ml. The phenylosazones were prepared at pH 5 (Edson, 1940), recrystallized from 30% (v/v) aqueous ethanol and dried *in vacuo* at room temperature.

RESULTS

Substrate specificity and rates of polyol oxidation

The rate of oxidation of a substrate in the complete dehydrogenase system was followed manometrically by measurement of oxygen uptake under the conditions of Blakley (1951) with methylene blue as a hydrogen carrier. In these experiments (Table 1) the rate of oxygen uptake was almost linear in the first hour but declined slowly during the second hour. The enzyme preparation consumed a small amount of oxygen in the absence of substrate.

The substrate oxidized most rapidly is sorbitol (L-gulitol), but it is important to recall that L-iditol is oxidized at approximately the same rate (see Blakley, 1951). Allitol reacts at less than half this rate. The other hexitols (galactitol (dulcitol), D-gulitol, D-mannitol, D-talitol) and 6-deoxy-L-gulitol are not oxidized. Of the pentitols, xylitol is oxidized faster than ribitol; D- and L-arabitol are not attacked. In the heptitol series the compounds

Table 1. *Oxidation of polyols by rat-liver polyol dehydrogenase*

Each Warburg flask contained 1·5 ml. of enzyme solution, 214 μmoles of potassium phosphate buffer, pH 8·0, $2{\cdot}72 \times 10^{-1}$ μmole of DPN, 51·2 μmoles of substrate and 3·2 μmoles of methylene blue (added from the side bulb after equilibration). Total volume, 3·2 ml. Gas, air. Temp., 38°. Time, 1 hr. The inseal contained 0·2 ml. of 2N-NaOH.

Substrate	O_2-uptake (μmoles)
Nil	0·60
Erythritol	0·60
D-Arabitol	0·55
L-Arabitol	0·50
Ribitol	6·83
Xylitol	11·79
Allitol	6·26
Galactitol (dulcitol)	0·50
D-Gulitol (L-glucitol)	0·60
L-Gulitol (D-glucitol, sorbitol)	13·84
6-Deoxy-L-gulitol (1-deoxysorbitol)	0·50
D-Mannitol	0·50
D-Talitol	0·50
D-*manno*-D-*gala*-Heptitol (perseitol)	0·50
D-*manno*-D-*talo*-Heptitol (volemitol)	0·45
L-*gulo*-D-*gala*-Heptitol	7·28
L-*gulo*-D-*talo*-Heptitol (β-sedoheptitol)	12·10
*meso*Inositol	0·50
d-Inositol	0·60
l-Inositol	0·60
Scyllitol	0·60
l-Viburnitol	0·55

containing the L-*gulo* configuration are oxidized but those which lack this configuration (perseitol and volemitol) are not attacked. The following are not oxidized: erythritol and the cyclitols (*meso*-inositol, *d*- and *l*-inositol, scyllitol and *l*-viburnitol).

The relative rates of oxidation of sorbitol, allitol, ribitol and xylitol found in the manometric experiments were confirmed by spectrophotometric measurement of reduced DPN (Fig. 1).

Coenzyme specificity

When TPN is substituted for DPN the enzyme system does not attack polyols.

Relationship between oxygen consumption and sugar formation

Table 2 shows that the dehydrogenation catalysed by the enzyme in the presence of methylene blue and oxygen corresponds almost quantitatively to the equation: polyol $+ \frac{1}{2}O_2 =$ reducing sugar (ketose) $+ H_2O$. Reducing sugar was absent from controls in which substrates were omitted. Since accurate factors for the reducing powers of ketoses other than fructose were not available, the amount of each sugar formed was calculated on the assumption that its capacity for reducing the Somogyi reagent equals that of glucose.

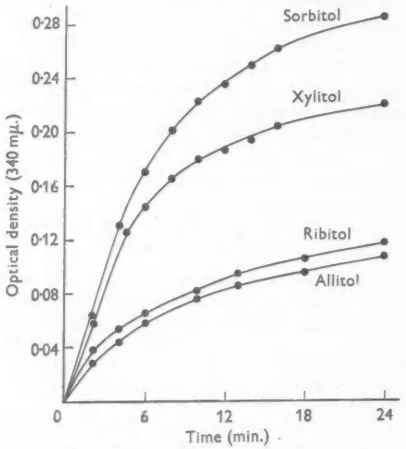


Fig. 1. Reduction of DPN by polyols in the presence of rat-liver enzyme. The reaction mixture (3·0 ml.) contained initially 1·0 ml. of enzyme solution, 100 μmoles of potassium phosphate buffer, pH 7·9, 9×10^{-2} μmole of DPN and 21·9 μmoles of substrate. Temp. 20°.

Table 2. *Formation of reducing sugar during polyol oxidation*

Warburg flasks contained the same quantities of enzyme and reagents as in Table 1. Gas, air. Temp. 38°. Time 3 hr.

Substrate	O_2-uptake* (μmoles)	Reducing sugar (μmoles)
Sorbitol	20·0	39·0
Allitol	6·8	12·6
Ribitol	8·5	15·5
Xylitol	15·7	29·7

* Oxygen uptake of the system without substrate has been subtracted.

Identification of the products of reaction

The isolation of sugar derivatives was restricted by the amount of substrate available and by the relatively poor yields of sugar due to progressive inactivation of the enzyme at 38°.

Allitol (60 mg.) formed 21 mg. of a reducing sugar which gave a red resorcinol reaction, a purple carbazole reaction and a yellow crystalline phenylosazone, m.p. (recryst.) 180–182·5° (decomp.). Levene & Jacobs (1910) reported the melting point of D-allosazone as 183–185° (decomp.) while Steiger & Reichstein (1935) gave 161–164° as the melting point of L-allosazone. The amount of osazone obtained was insufficient for measurement of optical rotation with the apparatus available, but the above evidence, considered in conjunction with the specificity of the enzyme, indicates that the product of allitol oxidation is D-allulose.

Ribitol (729 mg.) formed 165 mg. of reducing sugar giving a green resorcinol test and a purplish red carbazole reaction. The phenylosazone crystallized in yellow, flaky plates, m.p. (recryst.) 163–164°, $[\alpha]_D^{18} = -49{\cdot}8°$ in pyridine:absolute ethanol:2:3, v/v (*c*, 0·92). The osazone was identified as D-arabinosazone (cf. Reichstein, 1934) and the product of ribitol oxidation as D-ribulose.

Xylitol (729 mg.) formed 198 mg. of a reducing sugar which gave a green resorcinol reaction and a purplish red carbazole reaction. The phenylosazone crystallized as yellow rhombic needles, m.p. (recryst.) 158–160°, $[\alpha]_D^{18} = -38{\cdot}7°$ in absolute ethanol (*c*, 1·2). The osazone was identified as D-xylosazone and the product of xylitol oxidation as D-xylulose.

L-Gulo-D-gala-*heptitol* (18 mg.) formed a reducing sugar which gave a purplish red resorcinol test and a phenylosazone crystallizing in fine yellow needles, m.p. (recryst.) 197° (decomp.). The sugar migrated on a paper chromatogram at the same rate as perseulose.

β-Sedoheptitol (18 mg.) formed a reducing sugar which gave a purplish red resorcinol reaction. The phenylosazone crystallized in fine yellow needles, m.p. (recryst.) 194° (decomp.). The enzymically formed sedoheptulose gave one well-defined spot with the same R_F value as the chief spot in the reference mixture of sedoheptulose and sedoheptulosan (see also Noggle, 1953).

Reversibility of reactions

Blakley (1951) has shown that the oxidation of sorbitol and of L-iditol is reversible. The analogous oxidations of xylitol, L-*gulo*-D-*gala*-heptitol and *β*-sedoheptitol are also reversible (Fig. 2). D-Tagatose, D-mannoheptulose and D-glucoheptulose do not oxidize reduced DPN in the presence of the enzyme. There was no change in optical density at 340 mμ.

when these ketoses were added to an equilibrium mixture prepared from buffered enzyme, sorbitol and DPN.

The failure of the enzyme to reduce D-tagatose and D-*manno*heptulose is consistent with its failure to oxidize the corresponding epimeric pairs of polyols, namely, dulcitol and D-talitol, volemitol and perseitol; and from the absence of reaction with D-*gluco*heptulose it can be inferred that the enzyme will not oxidize *gluco-gulo*-heptitol or D-*gluco*-D-*ido*-heptitol (D-*ido*-L-*gulo*-heptitol).

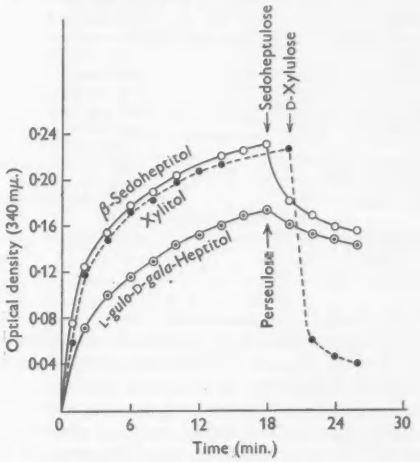


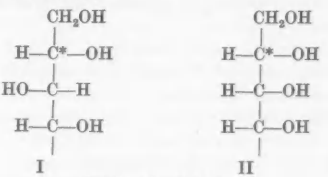
Fig. 2. Reversibility of polyol oxidation. The reaction mixture (3·2 ml.) contained initially 1·0 ml. of enzyme solution, 100 μmoles of potassium phosphate buffer, pH 7·9, 9×10^{-2} μmole of DPN, and 20 μmoles of polyol. Temp. 20°. Ketoses (20 μmoles) were added at points indicated by arrows. The experiment with xylitol was done with a dilute solution of very active sheep-liver enzyme (Todd, 1954); the other experiments with rat-liver enzyme.

DISCUSSION

Rat-liver polyol dehydrogenase catalyses a general reaction:

acyclic polyol + $DPN^+ \rightleftharpoons$ ketose + DPNH + H^+,

if the stereochemical pattern of the substrate conforms to the specific requirements of the apoenzyme. Comparison of the structures of the polyols listed in Table 3 will reveal that the vulnerable configurations are I and II:



* Site of oxidation.

The oxidation of fully hydroxylated acyclic polyols having five to seven carbon atoms will not occur unless the following conditions are satisfied. (1) The secondary alcohol group involved in the oxidation must be adjacent to a primary alcohol group and its hydroxyl must possess an L configuration with respect to this primary alcohol group. The need for a primary alcohol group is indicated by the inability of the enzyme to oxidize glucose, gluconate (Blakley, 1951) and 6-deoxy-L-gulitol. (2) The hydroxyl of the secondary alcohol group next but one to the oxidizable group must also possess an L configuration. (3) The configuration of the intervening secondary hydroxyl may be either D or L. In this connexion it may be significant that most substrates possessing configuration I (sorbitol, L-iditol, xylitol, β-sedoheptitol) are oxidized more rapidly than those having configuration II (allitol, ribitol). The relative rates of oxidation, referred to sorbitol as 100, are: sorbitol, 100 > L-iditol, 96 (Blakley, 1951) > β-sedoheptitol, 87 > xylitol, 85 > L-*gulo*-D-*gala* heptitol, 52 > ribitol, 49 > allitol, 45. The specificity rules based on the evidence at hand do not exclude the possibility that certain ω-deoxy polyols (e.g. 6-deoxy-D-iditol, 6-deoxy-L-glucitol) will be attacked by the liver enzyme in a manner analogous to the L-fucitol oxidation of *Acetobacter suboxydans* (Richtmyer, Stewart & Hudson, 1950).

It is proposed to call the enzyme L-*iditol dehydrogenase* because L-iditol is an unambiguous substrate containing the specific configuration at both ends of the polyol chain. The enzyme occurs in all mammalian livers so far examined (Blakley, 1951; Todd, 1954), and may be widely distributed in micro-organisms and plants (McCorkindale, 1953; Edson, 1953).

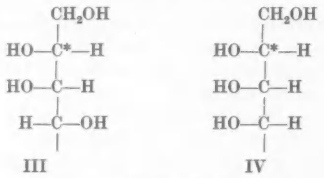
The specificity can be explained in terms of the polyaffinity theory (Bergmann, 1935–6; Gottschalk, 1950) by assuming that oxidizable substrates are attached to the apoenzyme and definitely oriented on its surface by the formation of three or four hydrogen bonds. The stereochemical mechanism of the dehydrogenation has already been discussed in a preliminary way (Edson, 1953).

The specificity of L-iditol dehydrogenase is different from that exhibited by *Acetobacter* grown in cultures at pH 5–6·8. Bertrand (1904) discovered the selective oxidation of polyols by *A. xylinum* and described the phenomena in a well-known rule. Subsequent observations on *A. suboxydans*, too numerous and well-known to be cited in detail here, led to the confirmation of Bertrand's rule by Kluyver, Underkofler, Reichstein, Hudson, Richtmyer and others, and to its extension (Hann, Tilden & Hudson, 1938; Richtmyer *et al.* 1950; Stewart, Richtmyer & Hudson, 1952; Pratt, Richtmyer & Hudson, 1952).

Table 3. *Comparison of polyol oxidation by* Acetobacter suboxydans *and liver polyol dehydrogenase*

Substrate	Ketose formed	
	Cultures of *A. suboxydans*	Liver polyol dehydrogenase
Erythritol	L-Erythrulose	Not oxidized
D-Arabitol	D-Xylulose	Not oxidized
L-Arabitol	Not oxidized	Not oxidized
Ribitol	L-Ribulose	D-Ribulose
Xylitol	Not oxidized	D-Xylulose
Allitol	L-Allulose	D-Allulose
Galactitol (dulcitol)	Not oxidized	Not oxidized
L-Gulitol (D-glucitol, sorbitol)	L-Sorbose	D-Fructose
D-Gulitol (L-glucitol)	Not oxidized	Not oxidized
6-Deoxy-L-gulitol (1-deoxy-D-glucitol)	6-Deoxy-L-sorbose	Not oxidized
D-Iditol	Not oxidized	Not oxidized
L-Iditol	Not oxidized	L-Sorbose
D-Mannitol	D-Fructose	Not oxidized
D-Talitol (D-altritol)	D-Tagatose	Not oxidized
L-*gulo*-D-*gala*-Heptitol (L-*gala*-D-*gluco*-heptitol)	Not oxidized	L-*gala*Heptulose (perseulose)
L-*gulo*-D-*talo*-Heptitol (D-*altro*-D-*gluco*-heptitol, β-sedoheptitol)	L-*gulo*Heptulose	D-*altro*Heptulose (sedoheptulose)
D-*manno*-D-*gala*-Heptitol (L-*gala*-D-*manno*-heptitol, perseitol)	L-*gala*Heptulose	Not oxidized
D-*manno*-D-*talo*-Heptitol (D-*altro*-D-*manno*-heptitol, volemitol)	D-*manno*Heptulose and sedoheptulose (approximately equal parts)	Not oxidized

The Bertrand-Hudson rule states that acyclic polyols possessing configuration III or IV are oxidized to ketoses by *A. suboxydans*.



* Site of oxidation.

The hydroxyl of the secondary alcohol group involved in the oxidation and the *cis*-vicinal secondary hydroxyl must have a D configuration with respect to the primary alcohol group (or the equivalent suggested by Richtmyer *et al.* (1950) in certain ω-deoxy polyols) adjacent to the site of oxidation.

Table 3 shows a comparison of the sugars formed during the oxidation of polyols by *A. suboxydans* and the L-iditol dehydrogenase of rat liver. The contrast in specificity is readily apparent. The behaviour of *A. suboxydans* suggests that it contains a polyol dehydrogenase which also requires multipoint, but spatially different, attachment of the substrate. Since D-mannitol is a typical and unambiguous substrate for *A. suboxydans*, it is proposed to call the enzyme responsible for reactions described in the Bertrand-Hudson rule, D-*mannitol dehydrogenase*. It is a DPN-linked soluble dehydrogenase which can be extracted from *A. suboxydans* and other sources (Edson, 1953; Arcus & Edson, 1954). Its properties will be described in a later communication. Dulcitol, which is not attacked by either D-mannitol or L-iditol dehydrogenase, is well known to be metabolized by certain micro-organisms. This fact suggests the existence of a third type of DPN-linked enzyme, *galactitol dehydrogenase*, specific for the stereochemical pattern of dulcitol. A soluble enzyme of this kind oxidizing dulcitol to tagatose has been extracted from micro-organisms (Shaw, 1954).

Thus an examination of the specificity of rat-liver polyol dehydrogenase has led to the hypothesis that the liver enzyme is merely one member of a group of at least three DPN-linked polyol dehydrogenases, each of which is highly specific for a particular stereochemical pattern. The existence of these enzymes will account for many facts known about the metabolism of polyols in different organisms and a study of their distribution and properties may ultimately explain the peculiar occurrence of polyhydric alcohols in plants (Edson, 1953).

SUMMARY

1. The polyol dehydrogenase of rat liver catalyses the reaction:

acyclic polyol + DPN^+ ⇌ ketose + DPNH + H^+,

with a high degree of stereochemical specificity. It does not attack cyclitols.

2. The specificity of the enzyme is described by a rule which is contrasted with the Bertrand-Hudson rule for *Acetobacter suboxydans*. A suitable name for the liver enzyme is L-iditol dehydrogenase.

3. Consideration of the metabolism of polyols in different organisms leads to the conclusion that there are at least three types of polyol dehydrogenases (L-iditol, D-mannitol and galactitol dehydrogenases) distinguishable by their stereochemical affinities.

We wish to thank the persons named above who have generously given us samples of rare materials, especially Dr N. K. Richtmyer whose courtesy made it possible for this study to be reasonably complete; and we are indebted to Prof. L. H. Briggs (Auckland University College) for use of high-pressure hydrogenation facilities in his laboratory.

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A Sodium-Yeast and some of its Properties

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An observation made in this laboratory by Winder (1950) showed that if baker's yeast (*Saccharomyces cerevisiae*) be allowed to ferment in the presence of sodium citrate and glucose there is a large entrance of sodium ions into the cell. It appeared, therefore, that it would be possible to prepare a 'sodium-yeast', i.e. a yeast in which all or nearly all the potassium would be replaced by sodium, such a yeast being analogous to the 'ammonium-yeast' described by Conway, O'Brien & Boyle (1941) and Conway & Breen (1945). A comparison of the properties of such a yeast with those of a control 'potassium-yeast' and with those of the ammonium yeast might well throw light on the role of these ions in metabolic processes. It proved possible by the methods here described to prepare, as required, a yeast in which 98% of the cellular potassium was replaced by sodium and to measure its growth, fermentation and oxygen consumption rates.

METHODS

Preparation of a sodium-yeast. A fresh twice-washed sample of baker's yeast (usually 10 g. fresh wt.) was used; 1 vol. of the centrifuged material was suspended in 20 vol. of a medium containing 5% (w/v) glucose and 0·2 M sodium citrate and fermentation was allowed to proceed for 2 hr. The yeast was then centrifuged, washed with 20 vol. of distilled water and resuspended in fresh fermentation medium. This procedure was repeated until the K content of the yeast was reduced as far as possible. As a control, a sample of the same fresh yeast was treated similarly, using a medium containing potassium citrate instead of the sodium salt. The yeast which has been treated in this way is referred to as 'K-yeast'. Whenever an analysis was to be carried out the yeast was twice washed with 20 vol. of distilled water to remove Na or K from the interspaces.

The fermentations were carried out under three different sets of conditions: (*a*) at room temperature with occasional stirring, (*b*) at 37° with occasional stirring, (*c*) at room temperature with continuous shaking. In the final stages of

preparation of an Na-yeast K was taken up so readily that the flasks in which the fermentation was carried out and the centrifuge cups used had to be coated on the inside with paraffin wax.

Sodium and potassium analyses. These were carried out with a flame photometer using an internal Li standard. The procedure consisted in taking a sample of twice-washed yeast, boiling in 10–20 vol. of distilled water and diluting as required to obtain a convenient reading on the instrument. Centrifuging the solutions before atomizing in the photometer appeared to make no difference to the results and the practice was not maintained.

Growth experiments. The medium for Na-yeast was 2·6 g. glucose, 0·57 g. NaH_2PO_4, $2H_2O$, 0·4 g. $(NH_4)_2SO_4$, 0·1 g. $MgSO_4$, $7H_2O$, 1 mg. thiamine, 1 mg. *p*-aminobenzoic acid, 2 mg. riboflavin, 2 mg. nicotinic acid, 2 mg. calcium pantothenate, 4 mg. pyridoxine hydrochloride, 0·05 mg. inositol and 0·004 mg. biotin, made up with water to 100 ml.

For K-yeast the medium was the same as above but an equivalent amount of KH_2PO_4 was substituted for the NaH_2PO_4. The pH of such media was 3·6. In investigating the effect of added K on the growth rate of Na-yeast varying amounts of KCl were incorporated in the medium.

In each growth experiment 0·1 ml. of a 1:100 suspension of the yeast was added to 100 ml. of sterile growth medium in a Pasteur flask and incubated at 28°. Such an inoculum contained approximately 100–400 cells. Every 12 or 24 hr. two small samples were withdrawn from the medium after shaking and a count made on each with a haemocytometer, the mean being used to plot the result. Sterile procedures were used throughout.

Fermentation experiments. The microdiffusion procedure described by Conway (1950) was followed. 1 ml. of 1:100 yeast suspension + 1 ml. of 5% (w/v) glucose were placed in the outer chamber and the CO_2 evolved was absorbed in 1 ml. of 0·1N-NaOH containing a mixed phenolphthalein and thymolphthalein indicator. After the requisite time, 0·5 ml. of saturated $BaCl_2$ was added and a back titration was carried out with 0·05N-HCl. Blanks in which the glucose was omitted were run for the same times in each case. All units were set up in duplicate or in triplicate.

In investigating the effect of the B-group of vitamins on the fermentation rate the following substances were incorporated in the 5% glucose solution (amounts in mg./100 ml.): pyridoxine hydrochloride 4, nicotinic acid 2, riboflavin 2, calcium pantothenate 2, thiamine 1, *p*-aminobenzoic acid 1, inositol 0·05 and biotin 0·0001. The procedure with this fermentation medium was the same as above.

Oxygen consumption. Oxygen consumptions were measured by the Warburg manometric method at 30° and 120 strokes/min. 3 ml. of a 1:100 suspension of yeast were used. The suspending medium had the following composition: 0·095% Na_2HPO_4, 0·080% NaH_2PO_4, 0·6% NaCl. The pH of the suspension was 6·63. No glucose was added.

RESULTS

The sodium and potassium content of the sodium-yeast preparations

The effects of seven fermentations (2 hr. periods) at room temperature in the sodium citrate medium on the Na and K content of a yeast sample are shown in Fig. 1. After one fermentation of 2 hr. the K content of the yeast is reduced to about half its normal value and after the first few fermentations the rate of replacement of K by Na becomes greatly reduced. In this sample the sum of the Na and K contents of the original fresh yeast in this example was 5·2 + 133·7 = 138·9 m-equiv./kg. wet yeast, the mean value for the sum of the two throughout being 140·7 m-equiv./kg., the figures ranging from 133·0 to 146·0, indicating very clearly that under these conditions there was an equivalent replacement of K^+ by Na^+.

For somewhat different conditions as listed in Table 1 the gain in Na^+ may be appreciably greater than the loss of K^+.

As has been mentioned under 'Methods', vigorous shaking at room temperature and a temperature of 37° were examined. The results were very similar but appear significant for the last traces of K remaining in the yeast. The raised temperature, or vigorous shaking at room temperature seem to reduce the K content more quickly to somewhat lower levels as shown in Table 1. Thus after thirteen to eighteen fermentations the mean K content under such conditions was 3·6 m-equiv./kg. of centrifuged yeast, whereas at room temperature with occasional stirring the mean value after twenty-one to twenty-two fermentations was 4·4 m-equiv./kg.

The effect of waxing the interior of the vessels. Under 'Methods' it was mentioned that waxing the interior of the vessels gave a greater K depletion of the yeast in the final stages. Table 2 shows the marked differences that result, indicating at the same time the effect of mere traces of K in the external medium.

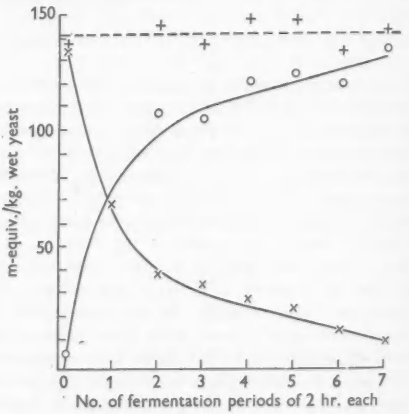

Fig. 1. Changes in the K (×) and Na (○) contents of a yeast sample fermented for seven successive 2 hr. periods in 5% glucose + 0·2M sodium citrate. The dotted line represents the mean Na + K content throughout (+).

Table 1. *Sodium and potassium content of sodium-yeast preparations*

Preparation	No. of 2 hr. fermentation periods	Na content m-equiv./kg. wet yeast		K content m-equiv./kg. wet yeast	
		Before	After	Before	After
		Room temperature, occasional stirring			
I	7	—	—	138·7	9·5
II	7	5·2	133·7	133·3	8·0
III	21	—	160·9	—	3·0
IV	22	—	140·5	—	4·1
V	21	0·6	137·9	143·3	6·0
		Room temperature, vigorous shaking			
IX	6	2·4	198·5	144·7	22·5
X	16	2·4	184·9	144·7	4·4
XI	18	2·1	138·6	128·1	3·0
		At 37° with occasional stirring			
VI	12	5·0	134·8	148·1	8·3
VII	13	1·3	96·4	168·0	4·0
VIII	15	—	131·9	162·8	3·0

Table 2. *The effect of coating the fermentation flasks and centrifuge cups with paraffin wax on the final potassium concentration in the yeast*

No. of fermentation periods	K content of the yeast in m-equiv./kg. wet yeast			
	Unwaxed	Waxed	Unwaxed	Waxed
0	122·7	—	154·1	—
7	23·5	25·2	22·0	11·4
8	21·1	—	21·4	—
9	19·2	16·0	17·3	—
10	19·0	14·7	17·6	5·8
11	15·1	—	—	—
12	13·9	—	15·2	—
13	12·8	5·7	16·0	—
15	—	—	—	3·6
21	—	—	—	3·0

Table 3. *Replacement of sodium of a sodium-yeast by potassium*

	m-equiv./kg. wet yeast	
	Na	K
I. Fresh yeast	2·1	128·1
II. Sodium-yeast prepared from I	141·6	4·1
III. After 2 hr. fermentation of sample II in potassium citrate solution	48·4	144·3
IV. Sample II after two 2 hr. fermentation periods	5·9	173·1

In the investigation of growth, fermentation and oxygen consumption rates described below, only samples of 'Na-yeast' with the lowest K levels were used.

Reversal of the process. If a 1:20 suspension of Na-yeast be allowed to ferment as described in the preparative method for 2 hr. in 5% (w/v) glucose + 0·4 M potassium citrate, K is found to enter very rapidly, replacing the Na^+. Two such fermentation periods are sufficient to restore the Na–K balance to normal levels. Results of such an experiment are given in Table 3.

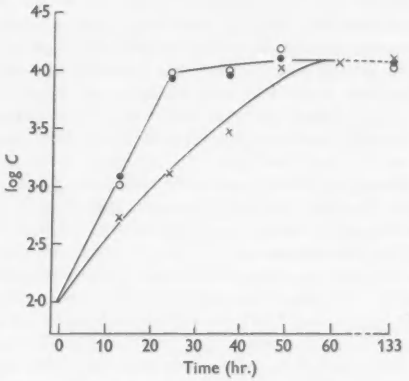

Fig. 2. Growth curves of normal (●), sodium (×) and potassium (○) yeasts at 28° in a low-K medium. C = cells/cu.mm. (The count at zero time is taken as 100 per cu.mm.)

Growth curves for the sodium-yeast

Growth experiments were carried out as described under Methods. Normal, Na- and K-yeasts were grown in the low-potassium medium already described, which had a potassium content of

0·55 mg./100 ml. derived from the added biotin solution which was available only in a phosphate buffer (British Drug Houses' preparation). It will be seen from Fig. 2 that the preparative method has of itself no effect on the growth rate of the yeast as the curves (log. (count) against time) for normal untreated yeast and for the K-yeast are identical. The growth of the Na-yeast is, however, much slower. It may be noted also that the maximum cell population attained is approximately the same in each case, but is approached more slowly in the case of Na-yeast.

In Fig. 3 an interesting observation is illustrated. When repeating such growth experiments with samples from another preparation, it was found that whereas the normal and K-yeast curves practically overlap, as before, the Na-yeast obviously did not begin to grow for more than 24 hr. and the final level was much lower than that of the other curves. Examination of the Na-yeast sample from which the inoculum was made in this case revealed that it was almost entirely in a spore form. No reason for this difference can be suggested as the yeast samples were in all cases prepared and stored under exactly the same conditions. The experiment described below would seem to indicate that during these 24 hr. the yeast was accumulating K^+ from the medium in which it was present in low concentration. Microscopic examination after 60 hr. growth failed to reveal any spore forms.

The effect of added potassium on growth of sodium-yeast. The Na yeast sample used in this experiment was the spore form mentioned above. The growth medium was the same as before, but for samples *A* and *B* potassium was added in amounts as indicated on Fig. 4. Curve *C* has been transferred from Fig. 3 for comparison. It is seen that in the presence of the added K the samples grow from the beginning, and with the higher K content a somewhat higher total cell population is finally reached.

Fermentation studies

A series of experiments on the rate of production of CO_2 by these yeasts is summarized in the graphs of Fig. 5. The Na-yeast and the control K-yeast give practically the same rate which is only about half of that of the normal untreated yeast. The rate for K-yeast is slightly higher than that for Na-yeast but by an amount so small as to be scarcely significant. In order to test the idea that this reduction in the fermentation rate following the preparative method used might be due to loss of coenzyme factors from the yeast, the fermentation studies were repeated incorporating vitamins of the B-complex in the medium as described under 'Methods'. No significant difference from the curves illustrated was observed.

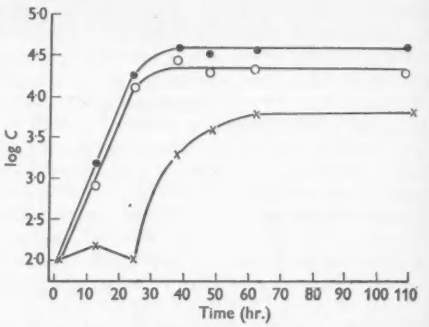

Fig. 3. Growth curves of further samples of normal (●), sodium (×) and potassium (○) yeasts at 28° in a low-K medium from spore inoculum (see text).

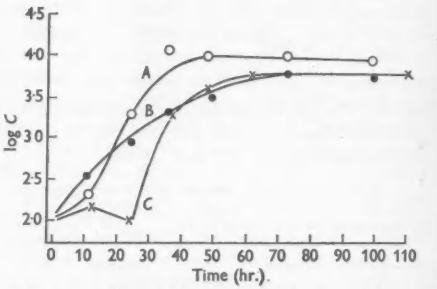

Fig. 4. Effect of added K on the growth rates of Na-yeast. Curve *A*, growth in a medium containing 40·04 mg. K/100 ml.; curve *B*, 0·94 mg. K/100 ml.; curve *C*, 0·55 mg. K/100 ml. This last curve has been transferred from Fig. 3 for comparison. Temp. 28°.

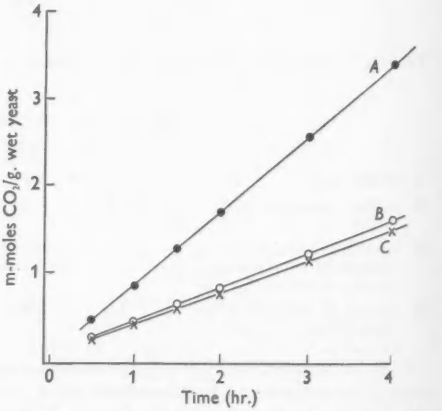

Fig. 5. Fermentation rates measured by microdiffusion method for CO_2 evolution the small blank values being subtracted. *A* (3 expts.), normal yeast; *B* (4) K-yeast; and *C* (5) Na-yeast.

Table 4. *Summary of O_2 consumption experiments*

Sample no.	Oxygen uptake (μl./hr./30 mg. centrifuged yeast) Fresh yeast	Na-yeast	K-yeast
4	46·1	29·5	61·6
5	20·4	17·1	62·3
6	39·3	63·0	—
7	45·3	—	—
8	52·0	—	—
9	43·0	—	—
10	41·5	31·8	122·5
13	—	—	62·8
14*a*	—	10·6	99·9
14*b*	—	13·3	108·1
Mean	41·7 (7)	27·6 (6)	86·2 (6)
Mean dry wt. (mg.)	6·32	6·84	6·98
Mean Q_{O_2}	−6·5	−4·0	−12·35

Oxygen consumption rates

The oxygen consumptions of the resting yeast samples showed a linear relation with time and are summarized in Table 4. The rate for Na-yeast is only about two-thirds that of the rate for normal fresh yeast, while that for K-yeast is about twice the latter. The difference between the K- and Na-yeast rates is very striking, the former being more than 3 times the latter.

Physical characteristics

Samples of each yeast were examined microscopically. The K-yeast showed no visible difference from the normal fresh yeast. All the cells were clear, oval and of about the same size in each case, indicating that the preparative method, the repeated fermentations and washings and the effect of the high external concentration of the potassium citrate produced no apparent morphological changes when so examined. The Na-yeast cells, on the other hand, were more opaque to light and contained more clearly distinguishable granular material. They had a smaller average size and a much more irregular, almost angular, outline than in the other two samples.

Also, on centrifuging during the preparative stages the Na-yeast always packed more closely and occupied a smaller volume than the corresponding K-yeast being centrifuged at the same time. (In the various studies here described the amount of yeast refers to the centrifuged material. The average dry weights of 30 mg. of centrifuged Na-yeast and K-yeast were very similar, being 6·84 and 6·90 mg. respectively.)

It was also noticed that if samples of the two yeasts were left exposed to air, the Na-yeast dried much more quickly, becoming rapidly reduced to a tough glue-like consistency.

DISCUSSION

The fact that waxing the interior of the fermentation and centrifuging vessels allows of a very appreciable ultimate reduction of the K content of the Na-yeast (which could be finally reduced to about 3 m-equiv. K/kg. centrifuged yeast 98 % of the cellular K^+ being lost and replaced by Na^+) suggests that rigorous purification of the sodium citrate from traces of K may give a greater K depletion.

Pending further possible reduction in the K values results of interest appear in the comparison between the Na-yeast and a preparation to which K^+ is added and one treated in a similar manner but with potassium citrate instead of sodium citrate.

From the results it is evident that there is no appreciable difference in the fermentation rates when K is replaced by Na. There are, however, large differences in oxygen consumption and in growth rates and also in the physical characteristics of the Na-yeast. There are many observations in the literature concerning effects of K^+, NH_4^+ and Na^+ on enzyme actions. Here the subject is confined to the yeast cell. Using quickly dried yeast, Meyerhof & Kaplan (1951) found for instance that Na^+ alone is unable to allow fermentation but is slightly harmful if enough K^+ and NH_4^+ are present—an observation that does not apply to the living yeast cells investigated here.

Similar investigations were previously carried out on an ammonium-yeast in which practically all the K^+ was replaced by NH_4^+ (Conway & Breen, 1945). It is of interest to compare the results with the Na-yeast.

The substitution of K by NH_4 caused a marked decrease in growth rate, while fermentation rate was decreased to 40 %. Also the substitution of K ions by NH_4^+ does not decrease the oxygen consumption but markedly increases it.

It is true that in the Na-yeasts examined there is a small concentration of K^+ present and this small concentration may be significant in action. But where large differences in oxygen consumption and growth rates exist, a further depletion of K ions may be expected only to increase such differences somewhat further. On the other hand, a further K depletion may show effects on fermentation rates which are not now apparent.

SUMMARY

1. A method is described for replacing up to 98 % of the cellular potassium of yeast by sodium, thus forming a 'sodium-yeast' analogous to the ammonium-yeast previously described.

2. The process can be easily and rapidly reversed.

3. The 'sodium-yeast' grows in a medium containing as little as 0·55 mg. K/100 ml. but at a much slower rate than a control 'potassium-yeast'.

4. The fermentation rate does not differ significantly from that of a control 'potassium-yeast'.

5. The resting oxygen consumption rate is only 32% of that of the control 'potassium-yeast' and about 62% of that of a fresh yeast sample.

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